Host and molecular mechanisms behind the persistence of equine strangles

Luke McLinden, BVMedSci (Hons)

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Dedication

To Dustin, for reminding me why I am studying veterinary medicine, how important this field can be and how it can be a vehicle for improving the health and welfare of animals.

To Yona, for starting this whole journey.

To Grandad, for keeping me curious.

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Abstract

Background: Equine strangles is a widespread and highly infectious disease caused by the bacterium *Streptococcus equi* subspecies *equi* (*S. equi*). Despite the long history of strangles, much is still unknown about *S. equi* and the disease it causes. Understanding the complex interplay between host and bacterial factors that contribute to the carrier state is crucial for effective management of equine strangles. The aims of this project were to 1) evaluate the effectiveness of a strangles screening protocol at a UK welfare centre equids and 2) use nanopore sequencing alongside bioinformatic analysis to investigate structural variants in acute and persistent *S. equi* isolates

Methods: Phase 1: The clinical records of 626 equids admitted to a UK welfare centre between 2017 and 2021 were analysed. Admitted equids were subject to a strangles screening process consisting of paired dual target iELISA serological tests and guttural pouch endoscopy and lavage. Phase 2: The genomes of 11 acute and persistent *S. equi* isolates were analysed using nanopore sequencing technologies to investigate structural variants.

Results: Phase 1: Retrospective analysis of the clinical records of admitted equids found that the most effective way to diagnose strangles was through guttural pouch endoscopy and lavage. No host factors or haematological parameters were significantly associated with strangles carriage. The dual target iELISA was found to be unreliable for the purpose of carrier detection. Phase 2: Deletions and inversions were found in key genomic regions including genes encoding the hyaluronic acid capsule and key sortase-processed cell surface proteins. A prophage was also found, integrated within genes encoding for the hyaluronic acid capsule.

Discussion: These findings highlight the dynamic nature of persistence within the guttural pouch and reinforce the importance of implementing effective strangles screening protocols to prevent future outbreaks and maintain strangles-free herds. Endoscopically guided guttural pouch lavage and quantitative PCR was confirmed to be the most effective method for carrier detection. *S. zooepidemicus* was found to cause persistent strangles-like disease and should increasingly be considered alongside *S. equi* when dealing with strangles. Evidence of genomic decay was identified, and the inversions observed could lead to the

development of subpopulations and represent a molecular mechanism associated with *S. equi* persistence. Structural variants could explain the failure of the dual-target iELISA.

1 Introduction: the persistence of strangles

1.1 Aims and objectives

The first aim of this project was to evaluate the effectiveness of a strangles screening protocol at a UK equid welfare centre (Bransby Horses, UK); this included investigating the efficacy of the current screening tests and identifying host risk factors associated with strangles carriage. This retrospective clinical study is the focus of Chapter Two 'The screening of strangles', following the review of literature in Chapter One 'The persistence of strangles'.

The second aim of this project was to use nanopore sequencing alongside bioinformatic analysis to investigate the role and presence of structural variants in persistent *S. equi* isolates. This study is the focus of Chapter Three 'The *Streptococcus* of strangles'; following this the final chapter 'Understanding the persistence of strangles' will draw on all previous chapters to discuss how the findings of this thesis relate to the wider context of strangles persistence and management.

The objectives of this project were to:

1. Determine the prevalence of *S. equi* in equids admitted to a UK rescue centre using retrospective data from clinical data and diagnostic results admitted between 2017 and 2021.
2. Identify risk factors associated with acute infection and the establishment of carrier state in a rescue centre population. Insight into host and pathogenic factors associated with severe disease and carrier states will be revealed through analysis of clinical data and diagnostic results from admitted equids.
3. Determine the effectiveness of the current screening tests and programme at a UK rescue centre by reviewing clinical records and determining the cause of any outbreaks of respiratory disease.
4. Investigate the epidemiology of *S. equi* by evaluating genomic decay and inversion sequencing in carrier isolates using nanopore sequencing and bioinformatic analysis.

1.2 Background

1.2.1 Equine strangles

Strangles is a bacterial infection of equids caused by *Streptococcus equi* subspecies *equi* (*S. equi*) (Van de Kolk and Kroeze, 2013); domestic equids comprise horses, donkeys and their hybrids, whereas non-domestic equids include zebras and wild asses (Janssen and Allen, 2015). Infection with *S. equi* is typically characterised by the acute onset of a fever, followed by mucopurulent nasal discharge and lymphadenopathy as the bacteria translocates across the mucosal epithelium (Boyle *et al.*, 2018). The name strangles is a result of the dyspnoea and dysphagia caused by abscessation of lymph nodes in the head and neck (Waller, 2014).

A clinically identical condition, presumed to be strangles, was first described in 1256 (Ruffo, 1256), although the disease and its causative organism *S. equi*, first identified by Schütz (1888), have likely been around for much longer. In the 17th century, strangles was considered an inevitability; indeed, it was suggested that the disease was transmitted *in utero* due to the high numbers of horses that contracted the infection across varied backgrounds, genetic profiles, and management systems (Solleysel, 1664, Paillot *et al.*, 2017). By the 20th century, risk factors had been determined (Todd, 1910), including age and body condition; however, these are still in dispute (Ling *et al.*, 2011). Management-related factors are commonly the only reliable association with outbreaks (Libardoni *et al.*, 2016, Laing *et al.*, 2021).

As was long suspected (George *et al.*, 1983) and later confirmed (Newton *et al.*, 1997a, Timoney *et al.*, 1998), *S. equi* has the potential to persist, in the guttural pouch, without causing clinical disease in a proportion of animals. *S. equi* has the potential to survive in this low nutrient state, intermittently shedding into the environment, allowing the organism to spread to naïve individuals; indeed, its success as a pathogen can be attributed to the ability to cause both acute and persistent disease. Chronically infected equids rarely show clinical signs, presenting a major obstacle to the prevention and control of outbreaks (Verheyen *et al.*, 2000). The challenges associated with detecting carriers are a key reason for the perpetual spread of *S. equi* (Pringle *et al.*, 2020b).

Strangles is endemic worldwide, with only Iceland remaining free from the disease, due to a self-imposed import ban of equids and geographical isolation (Björnsdóttir *et al.*, 2017).

Although this has created a strangles-free population, one result of this ban is the production of a naïve and potentially vulnerable population, as highlighted by an outbreak of *Streptococcus equi subspecies zooepidemicus (S. zooepidemicus)* there in 2010 (Björnsdóttir *et al.*, 2017). Outbreaks of strangles from across the globe have been analysed, with isolates sequenced using core genome multi locus sequence typing methods, revealing the genetic lineage of the most prevalent strains of *S. equi* (Mitchell *et al.*, 2021). The genetic diversity of circulating isolates highlights the need for targeted vaccines, diagnostics and therapies depending on the genetic basis of the afflicting strains of *S. equi*.

1.2.2 The *Streptococcus* of strangles

S. equi is a beta-haemolytic streptococcus in Lancefield group C (Schütz, 1888). *S. equi* is a host-restricted pathogen of equids, thought to have evolved from the commensal, and occasionally opportunistic pathogen, *S. zooepidemicus* (Waller *et al.*, 2011, Holden *et al.*, 2009, Harris *et al.*, 2015). *S. equi* and *S. zooepidemicus* can be differentiated by their differing ability to ferment lactose, sorbitol, and trehalose (Reed *et al.*, 2018).

Infection with *S. equi* is characterised by acute disease, succeeded by long-term infection; *S. equi* can persist in the guttural pouch, often in chondroids, for years without causing clinical signs (Newton *et al.*, 1997a). *S. equi* survives in this low nutrient state, intermittently shedding into the environment; indeed, its success as a pathogen can be attributed to the ability to cause both acute and persistent disease allowing the organism to be spread to naïve individuals.

Although strangles has been described since at least the 13th century (Ruffo, 1256), genomic analysis of a global collection of strains revealed a shared common ancestor around the turn of the 20th century (Harris *et al.*, 2015). This was a time when equids were used extensively for transport and in global armed conflicts, with the First World War overseeing the mixing of equids on a scale hitherto unknown before; massive host mortality during this time likely created a genetic bottleneck through which a fitter strain emerged (Waller, 2016).

The international transmission of *S. equi,* as demonstrated by Mitchell *et al.* (2021), is in accordance with the first criterion of the World Organisation of Animal Health (WOAH) listing of terrestrial animal diseases. The other three criteria are demonstrated elsewhere

(Björnsdóttir *et al.*, 2017, Boyle *et al.*, 2018), thus it was recommended strangles be added to this listing.

1.2.3 The guttural pouch

The guttural pouches, *diverticular tubae auditivae*, are paired extensions of the eustachian tubes that communicate with the nasopharynx via the nasopharyngeal ostia. These openings are created by a medial fibrocartilaginous lamina and the lateral pharyngeal wall; they are oriented rostroventrally to facilitate drainage (Dyce *et al.*, 2018, Freeman and Hardy, 2018). In adult horses the guttural pouches have an approximate volume of 300 – 500 ml (Hinchcliff *et al.*, 2008). In the horse, *rectus capitis ventralis*, *longus capitis* and the median septum constitute the partition between the two guttural pouches (Sisson and Getty, 1975), each separated into a medial and lateral section by the stylohyoid bone so that the lateral compartment is approximately one third the capacity of the medial (Freeman and Hardy, 2018). The guttural pouch mucosa, lined with pseudostratified epithelia, contains goblet cells and is able to clear foreign substances; IgGa and IgA have been found in the mucosa, whereas IgGc and IgG have only been found in the surrounding lymphoid tissues (Manglai *et al.*, 2000).

Guttural pouches are anatomically unique to perissodactyls, including equids and tapirs, the South American forest mouse, and hyraxes (Hinchcliffe and Pye, 1969); although they are not present in the white rhinoceros suggesting their presence may not be dependent on phylogenetic status (Endo *et al.*, 1998). Potential functions of these paired diverticular include acting as a resonation chamber, reducing head weight, and improving flotation (Baptiste, 1997). It has also been suggested that this anatomical arrangement contributes to the cooling of the blood supplied to the brain, in particular the internal carotid arteries, to ensure adequate thermoregulation during exercise (Baptiste *et al.*, 2000).

The guttural pouches are clinically relevant in equids (Hardy and Leveile, 2003, Freeman, 2008, Weston Davis and Caniglia, 2015) situated in close proximity to a number of significant structures, including the *Plica neurovasculosa* and *Plica neuralis*, folds located caudally in the medial compartment (König *et al.*, 2010, Freeman and Hardy, 2018). The *Plica neurovasculosa* contains the sympathetic trunk, branches of the vagus nerve, the

cranial cervical ganglion and the internal carotid artery, whereas the *Plica neurovasculosa* contains the glossopharyngeal, vagus, accessory, and hypoglossal nerves (König *et al.*, 2010). Additionally, the facial and mandibular nerves run along the dorsal surface of each guttural pouch and the external carotid arteries run along the lateral compartment (König *et al.*, 2010). The retropharyngeal lymph nodes are intimately associated with the guttural pouches, indeed, their abscessation and lymphadenopathy is a common presentation of strangles in equids (Todd, 1910). Therefore, pathology of the guttural pouch has wide-ranging and potentially devastating consequences by affecting the aforementioned structures.

A number of clinical conditions can affect the guttural pouch of equids, one commonly described condition is guttural pouch empyema (Judy *et al.*, 1999, Hardy and Leveile, 2003)**:** when abscesses of the retropharyngeal lymph node rupture and subsequently drain into the guttural pouch. This typically occurs in infectious diseases of the upper respiratory tract that result in lymphoid hyperplasia, including strangles (Hardy and Leveile, 2003, Judy *et al.*, 1999). Other diseases include guttural tympany, affecting foals less than one year of age, and guttural pouch mycosis, a rare but devastating diagnosis with a 50% mortality rate in untreated horses (Freeman, 2008).

Examination of the guttural pouch is most effectively achieved using flexible endoscopy, yielding the most information on the internal structure of the guttural pouches, pharynx, larynx, and nasal passages (Weston Davis and Caniglia, 2015). This modality allows for lavage and biopsies to be taken; however, epistaxis can occur notably in the case of guttural pouch mycosis thus caution is advised (Hardy and Leveile, 2003). External palpation can assess enlargements, such as in tympany, but this can be painful for the affected animal. Radiography and computed tomography can be useful in revealing fluid, opaque structures such as chondroids, inspissated purulent material, and arthropathies (Tucker and Farrell, 2001, Hardy and Leveile, 2003).

1.2.4 Use of the concept ‘gold standard’ in veterinary diagnosis and treatment

There is a debate in veterinary medicine around the use of gold standard as it pertains to diagnostic and treatment options (Jones and Podolsky, 2015, Skipper *et al.*, 2021). It is

increasingly considered an unhelpful and simplistic term, not factoring in the financial and practical realities of clinical practice and unable to escape its history as a term derived from business and finance (Jones and Podolsky, 2015). Although having an ideal to strive towards is not in itself an issue, it should be acknowledged that optimal and gold standard care are not always equivalent; furthermore, feelings of guilt and shame from owners and veterinary professionals alike have been linked to its use (Skipper *et al.*, 2021).

To combat the implied hierarchical and predefined decision-making process as denoted by gold standard, models of shared-decision making are being identified as better alternatives (Elwyn *et al.*, 2012). Terms such as spectrum of care more accurately represent the clinical decision-making process making clinicians and owners, as well as negating the negative emotions gold standard may invoke (Skipper *et al.*, 2021). This review employs the term gold standard purely as it pertains to sensitivity and specificity of diagnostic methods, this ensures its use is not used subjectively or inappropriately.

1.3 Manifestation of equine strangles

1.3.1 Pathogenesis

Contact with infected equids represents the primary cause of strangles infection; although, *S. equi* has been shown to persist in the environment for up to 34 and 13 days in wet and dry sites, respectively, and environmental persistence is an additional source of contagion (Durham *et al.*, 2018). Equids become infected via the oronasal route, likely through ingestion of contaminated material (Boyle *et al.*, 2018). Upon entry, *S. equi* attaches to the crypt cells of the lingual and palatine tonsillar tissue, before translocating to the submandibular, and retropharyngeal lymph nodes; less commonly the parotid, submaxillary and cranial cervical lymph nodes are also involved (Timoney and Kumar, 2008). *S. equi* is present in the oropharynx transiently and within hours after initial exposure it is commonly not detected on the mucosa (Rendle *et al.*, 2021).

Virulence factors act to mitigate the effects of the host immune response: the hyaluronic acid capsule aids immune evasion (Woolcock, 1974), IgG endopeptidases are secreted to cleave antibodies (Lannergard and Guss, 2006), and antiphagocytic binding proteins such as Se18.9 are secreted (Tiwari *et al.*, 2007) Additionally, SeM surface proteins block immune activity by binding to fibrinogen and immunoglobulin (Timoney *et al.*, 1997, Meehan *et al.*, 2009). High morbidity is achieved through this antiphagocytic activity, resulting in intra and extracellular multiplication in tonsillar and lymphoid tissue, including regional lymph nodes (Timoney and Kumar, 2008). Additionally, *S. equi* can produce a microscopic biofilm with potential adhesive functions (Steward *et al.*, 2017) that may play a role in persistence.

If lymph node abscessation occurs, it is not visible until 3−5 days after their infiltration, as large numbers of neutrophils are attracted to the site through the interaction of complement-derived factors and pathogen-associated molecular patterns such as peptidoglycan (Muhktar and Timoney, 1988). The ability of *S. equi* to import iron has been linked to its growth within these abscesses, with the secreted molecule equibactin facilitating this acquisition (Heather *et al.*, 2008, Harris *et al.*, 2015). As these abscesses grow, developing thick fibrous capsules, they can obstruct the airway and cause dyspnoea and dysphagia hence the name strangles (Boyle *et al.*, 2018).

If abscesses occur they rupture into the airways, guttural pouches or through the skin 7–28 days after initial infection (Waller, 2014): abscesses of the retropharyngeal lymph nodes typically rupture into the guttural pouches, draining into the nasopharynx and subsequent nasal passages resulting in copious mucopurulent discharge. This drainage is important for the resolution of the infection and removal of bacteria and is exhibited by 50% of equids with guttural pouch empyema (Boyle *et al.*, 2018, Judy *et al.*, 1999).

S. equi typically commences shedding 1–2 days after the onset of pyrexia and persists for 2–3 weeks; equids remain infectious for over six weeks following the drying up of their purulent nasal discharge (Boyle *et al.*, 2018). About 10% of affected animals continue to shed intermittently for years following the resolution of clinical signs (Pringle *et al.*, 2020b, Newton *et al.*, 1997a).

Severity is dose-dependent with around 10,000 colony-forming units required to cause disease in a mature and immunocompetent equid (Boyle *et al.*, 2018). Increasing the number of colony-forming units will result in more severe disease and a shorter incubation period, which can vary from 1–28 days (Boyle *et al.*, 2018, Judy *et al.*, 1999). Further complications occur if the infection causes secondary immune-mediated disease or if *S. equi* affects lymphoid tissue beyond the head and neck (Delph *et al.*, 2019, Duffee *et al.*, 2015, Whelchel and Chaffin, 2009).

1.3.2 Acute *Streptococcus equi* infection

Strangles is characterised by sudden pyrexia, mucopurulent intermittent nasal discharge and the abscessation of the submandibular and retropharyngeal lymph nodes (Timoney *et al.*, 1998). These are the three most common clinical signs, consistent across historical literature, surveillance data and outbreak case studies (Christmann and Pink, 2017, McGlennon *et al.*, 2021, Solleysel, 1664). Less common clinical signs include dyspnoea, pharyngeal swelling, lethargy, inappetence, dysphagia, depression, and the presence of chondroids (Rendle *et al.*, 2021). Although strangles has a low mortality rate, severe swelling of abscesses in the lymph nodes can lead to significant inflammation, asphyxia and, ultimately, death.

Pyrexia, typically accompanied by lethargy, occurs 3−14 days after initial exposure and is persistent; rectal temperature measurements can exceed 42°C (Boyle *et al.*, 2018). Fever ordinarily precedes bacterial shedding by 1−2 days, thus identification of its onset can be paramount to isolating individuals and controlling outbreaks, alongside strict biosecurity protocols (Waller, 2014).

Lymphadenopathy typically occurs in the retropharyngeal and submandibular lymph nodes although the parotid, submaxillary and cranial cervical lymph nodes can also be involved (Timoney, 1993). As abscesses form, clinically visible as warm swellings, and subsequently rupture, empyema of the guttural pouch or upper respiratory tract can occur; intermittent expulsion of this thick highly infectious pus results in the unilateral nasal discharge and cough present in around 50% of horses with guttural pouch empyema (Judy *et al.*, 1999). Incomplete drainage of purulent material in the guttural pouch can lead to the persistence of viable populations of *S. equi*, commonly found in chondroids, inspissated purulent material (Hardy and Leveile, 2003, Whelchel and Chaffin, 2009, Newton *et al.*, 1997a).

Abscessation and pharyngitis can result in obstruction of the upper respiratory tract with the possible temporary laryngeal hemiplegia resulting in paralysis of the arytenoid cartilage contributing to this difficulty to breathe and feed (Boyle *et al.*, 2018). Parotid and retrobulbar abscesses can create large swellings around the orbit resulting in obstructed vision ocular swelling (Boyle *et al.*, 2018). Pharyngitis can be significant, often with concurrent nasal discharge, inappetence, dysphagia, a mucoid cough, and laryngeal-associated pain; endoscopy can reveal inflamed mucosa, pharyngeal compression, and lymphoid hyperplasia (Boyle *et al.*, 2018). Affected equids may stand with their heads in an abnormal, extended position (Waller, 2014).

Systemic and mucosal immune responses are evident 2−3 weeks post-infection, and this immunity wanes over time (Hamlen *et al.*, 1994, Galan and Timoney, 1985, Boyle *et al.*, 2018). Hamlen *et al.* (1994) showed that 75% of foals exposed to *S. equi* six months after recovering from strangles were protected from severe infection, corroborated by historical and contemporary literature (Todd, 1910, Boyle *et al.*, 2018), although no animals were completely protected from clinical signs. The use of antimicrobial therapy has been demonstrated to interfere with the persistence of humoral immunity (Pringle *et al.*, 2020a).

Neonates can derive protection from colostral antibodies from exposed dams, and, subsequently, IgA and IgG in milk confers some protection by coating the upper respiratory and oral mucosa until the time of weaning (Galan *et al.*, 1986). Individuals with residual immunity, such as equids that are older or vaccinated and foals with maternal antibodies, may develop a milder form of the disease with short-lived clinical signs, termed atypical strangles, although these animals can still shed *S. equi* to susceptible animals (Sheoran *et al.*, 1997, Prescott *et al.*, 1982).

1.3.3 Persistent *Streptococcus equi* infection

Once ruptured, abscesses of the retropharyngeal lymph nodes typically drain into the guttural pouches, resulting in guttural pouch empyema. Most strangles cases are cleared within six weeks, but some animals can enter a carrier state, continuing to shed *S. equi* following the apparent resolution of an acute infection (Newton *et al.*, 1997a, Newton *et al.*, 1997b). If the purulent material is not cleared and loses fluid, this can form chondroids over time; both empyema and chondroids can act as chronic reservoirs of *S. equi* (Newton *et al.*, 1997a, Judy *et al.*, 1999).

An average of 10% of infected individuals in an outbreak develop into carriers (Boyle *et al.*, 2018, Sweeney *et al.*, 2005); although, this figure may be an underestimate and is highly variable between outbreaks, with detection rates being limited by current diagnostic sensitivity (Pringle *et al.*, 2019). Carriers intermittently shed bacteria into the environment, leading to recurrence and perpetuation of strangles within their herd as well as transmission to naïve individuals (Mallicote, 2015).

There is no consensus on the question of what constitutes a carrier animal, and the answer can be argued from a number of different perspectives. The gross appearance of the guttural pouches demonstrates a spectrum of persistence, from a grossly normal but microbiologically active biofilm (Steward *et al.*, 2017) to the presence of purulent material or chondroids harbouring *S. equi*. Likewise, the chronicity of infection and presence of an observable immune response on a serological ELISA might determine the risk of infection propagation (Ivens and Pirie, 2021). The PCR status and associated cycle threshold (CT) number, indicative of bacterial load via an inverse relationship, is often used to identify

carriers, but this may be too simplistic in some cases as little is inferred about the viability and infectivity of the *S. equi* identified.

The following definition is proposed: a strangles carrier is an equid in which a viable population of *S. equi* persists and continues to shed, intermittently or continuously, following the apparent resolution of infection. These carriers can be divided into categories based on gross signs of infection (e.g., the presence of chondroids within the guttural pouch), immune status, serological status, chronicity of infection (e.g., short-term carriers that have recently been exposed and long-term carriers that have harboured *S. equi* for months/years) and bacterial load. For clinical practitioners, a division based on gross signs of infection may be most appropriate with carriers being categorised as symptomatic or asymptomatic accordingly.

1.3.4 Complications of *Streptococcus equi* infection

S. equi has the potential to spread haematogenously, via lymphatics, septic focus, or by direct aspiration of purulent material (Boyle, 2017). Common sites include the lung, mesentery, liver, spleen, kidney, and brain (Boyle *et al.*, 2018, Sweeney *et al.*, 1987); additional clinical signs are dependent on the location of abscesses. This presentation is known as metastatic or 'bastard' strangles and has been documented since the 17th century (Solleysel, 1664). The prevalence of these complications ranges from 2−28% across outbreaks (Spoormakers *et al.*, 2003, Sweeney *et al.*, 1987, Duffee *et al.*, 2015); metastatic abscessation has consistently been shown to increase mortality (Ford and Lokai, 1980). Additional clinical signs are dependent on the location of abscesses, including respiratory distress due to tracheal compression, intermittent pyrexia, colic, anorexia, and haematological disturbances, such as neutrophilia and hyperfibrinogenaemia (Boyle *et al.*, 2018, Duffee *et al.*, 2015). Hypothesised risk factors include an elevated infectious dose, increased virulence of the infective bacterial strain, and an insufficient host response (Spoormakers *et al.*, 2003). The early use of antibiotics has been suggested as a possible predisposition (Ramey, 2007), due to decreased stimulation of cell-mediated immunity, although clinical evidence to support this is lacking (Duffee *et al.*, 2015).

Purpura haemorrhagica is caused by a type three hypersensitivity reaction, resulting in a necrotising vasculitis secondary to immune-complex deposition (Whitlock *et al.*, 2019). Its presentation can vary from innocuous to a potentially fatal complication of a *S. equi* infection (Boyle *et al.*, 2018). *S. equi* infection is the most common cause of purpura haemorrhagica, but vaccination with M-protein-containing vaccines, other bacteria, viruses, and neoplasia can similarly result in purpura complexes and vasculitis (Mallicote, 2015). Clinical signs include subcutaneous pitting oedema of the distal limbs, head, and trunk; dermal necrosis, and sloughing; petechial or ecchymotic haemorrhage on mucosal surfaces; as well as general signs of vasculitis, including colic, myopathies, and respiratory distress (Mallicote, 2015, Boyle *et al.*, 2018). Small intestine intussusception has also been reported as an associated complication (Pusterla *et al.*, 2003).

Myopathies can be seen with *S. equi* infection, with three predominant presentations (Boyle *et al.*, 2018): muscle infarctions (Kaese *et al.*, 2005) and rhabdomyolysis with either acute myonecrosis or progressive atrophy (Sponseller *et al.*, 2005, Valberg *et al.*, 1996). Muscle infarctions are an extreme presentation of purpura haemorrhagica with immune-complex deposition and vasculitis within muscles leading to necrosis; this holds a guarded prognosis (Kaese *et al.*, 2005, Durward-Akhurst and Valberg, 2018, Boyle *et al.*, 2018). Acute rhabdomyolysis is thought to be caused by toxic shock syndrome or the direct toxic effects of *S. equi* infection, with *S. equi* visible in sections of the myonecrosis (Whitlock *et al.*, 2019). The presenting signs include stiffness progressing to recumbency, pitting oedema along the epaxial muscles, with marked elevations of creatinine kinase and aspartate aminotransferase (Sponseller *et al.*, 2005, Whitlock *et al.*, 2019). Rhabdomyolysis with progressive atrophy is thought to be immune-mediated, similar to purpura haemorrhagica and associated muscle infarctions (Whitlock *et al.*, 2019). This presentation has primarily been identified in quarter horses following *S. equi* exposure; notably some had a concurrent storage myopathy, but this was not the case in all affected horses (Durward-Akhurst *et al.*, 2016, Lewis *et al.*, 2007, Valberg *et al.*, 1996). Clinical signs include progressive weakness, muscle atrophy, elevated serum levels of creatinine kinase and aspartate aminotransferase, and fibrosis of blood vessels (Boyle *et al.*, 2018).

Other complications associated with strangles include anaemia, agalactia, meningitis, septic arthritis, and endocarditis (Boyle *et al.*, 2018).

1.4 Diagnosis of equine strangles

1.4.1 Diagnosis of acute *Streptococcus equi* infection

The diagnosis of the acute presentation of strangles relies on a thorough understanding of an animal's history, with particular respect to onset, management structures, and possible exposure, including history of travel, or new arrivals to the farm (Boyle *et al.*, 2018). The clinical signs typically associated with strangles are pyrexia, mucopurulent nasal discharge, and lymphadenopathy (McGlennon *et al.*, 2021); lethargy, pharyngitis, and respiratory signs, such as a cough or dysphagia may also be present (Boyle *et al.*, 2018). Clinical signs can be variable and non-specific, indeed not all animals develop clinical signs (Boyle *et al.*, 2018, Tscheschlok *et al.*, 2018). Nevertheless, they form a vital part of any clinical diagnosis, especially during an outbreak where testing of all affected individuals may not be necessary (Rendle *et al.*, 2021).

Many different diagnostic modalities can be useful in aiding the diagnosis of strangles, as well as its complications including diagnostic imaging and clinical pathology (Boyle *et al.*, 2018). Radiography can be used to investigate the involvement of lymph nodes, as well as fluid in the pharynx and guttural pouch (Van de Kolk and Kroeze, 2013); ultrasonography can be used to locate abdominal and thoracic abscesses during metastatic strangles (Southwood and Wilkins, 2015); and cytopathology can aid in diagnosis confirmation following the aspiration of abscesses (Reed *et al.*, 2018). Although variable, biochemistry typically reveals elevated levels of serum amyloid A and fibrinogen and can be useful for monitoring progress during chronic or complicated cases (Boyle *et al.*, 2018). Likewise, haematological evaluation cannot be relied upon, but leucocytosis, as well as a neutrophilic left shift, is characteristic of a severe or complicated infection with *S. equi* (Boyle *et al.*, 2018, Duffee *et al.*, 2015).

Historically, pathogen identification has relied on the culture of *S. equi* due to its low cost and wide availability (Waller, 2014). However, sensitivity can be as low as 30−40% (Lindahl *et al.*, 2013a, Boyle *et al.*, 2012, Pusterla *et al.*, 2021), and other beta-haemolytic Streptococci such as *S. zooepidemicus* and *Streptococcus dysgalactiae* subspecies *equisimilis* complicate interpretation. Bacterial culture may yield false negative results in early stages of an infection when there is low bacterial count, and there is an inherent delay in receiving results (Boyle *et al.*, 2018). Low bacterial shedding, the presence of host-produced growth inhibitors and poor sampling technique and sample site collection can also lead to apparent

negative results (Pusterla *et al.*, 2021). For these reasons, as well as concurrent advances in other pathogen identification techniques such as polymerase chain reaction (PCR) assays (Waller, 2014), bacterial culture is no longer being validated as the gold standard method of *S. equi* detection (Boyle *et al.*, 2018).

Advances in PCR (Webb *et al.*, 2013, Noll *et al.*, 2020, Willis *et al.*, 2021, Baverud *et al.*, 2007) and loop-mediated isothermal amplification (LAMP) assays (Boyle *et al.*, 2018) have improved the sensitivity and specificity of the detection of *S. equi* and these assays are now regarded as the gold standard (Boyle *et al.*, 2018). The first PCR assays for *S. equi* were designed to detect a part of the *SeM* DNA sequence (Timoney and Artiushin, 1997); however; a homologue of *SeM* is known to exist in *S. zooepidemicus* resulting in cross-reaction (Kelly *et al.*, 2006). In addition, *SeM* is variably found within the *S. equi* genome, particularly in persistently infective isolates (Chanter *et al.*, 2000, Harris *et al.*, 2015), limiting the diagnostic value of assays that only target *SeM*. Other formats detect different non-*SeM* sequences such as *SeeI*, a superantigen gene (Baverud *et al.*, 2007, Boyle *et al.*, 2016), *eqbE*, an equibactin gene (Webb *et al.*, 2013), as well as *sodA* (Baverud *et al.*, 2007) or have multiple gene targets such as the commercially available triplex assay, which targets two *S. equi* genes (SEQ_2190 and *eqbE*) and an internal control (Webb *et al.*, 2013).

Modern PCR formats, such as quantitative PCR or nested PCR, are up to 3 times more sensitive than bacterial culture (Noll *et al.*, 2020, Baverud *et al.*, 2007), and results can be obtained within hours of arriving at a laboratory (Boyle *et al.*, 2018). Real-time quantitative PCR assays offer greater sensitivity than traditional formats and, at present, are considered to be the gold standard method for *S. equi* detection (Webb *et al.*, 2013, Waller, 2014, Lindahl *et al.*, 2013a, Boyle *et al.*, 2018). Recently, nested PCR has been demonstrated to offer greater sensitivity and specificity than quantitative PCR, with a potential for less false negatives during low-shedding periods and no cross reaction with *S. zooepidemicus* (Noll *et al.*, 2020). Although this nested PCR assay evaluates *SeM*, it has been shown to not cross-react with *SzM*. *SeM* is variably found within the *S. equi* genome, particularly in persistently infective isolates (Chanter *et al.*, 2000, Harris *et al.*, 2015) so further evaluation of the utility of this assay is required before conclusions are reached. Nevertheless, this is an exciting development and may provide a superior testing format than traditional PCR.

Point-of-care PCR assays have also been recently validated with similar sensitivity and specificity results to quantitative PCR providing a conclusive result in less than an hour in most tested samples (Willis *et al.*, 2021). However, the point-of-care PCR assay had a lower limit of detection than, the limits being 277 and three *egbE* target genes respectively (Willis *et al.*, 2021); this is likely due to is faster extraction and purification processes. Willis *et al.* (2021) acknowledge the limitations of this assay, insisting that clinical signs consistent with strangles should result in strict movement restrictions and biosecurity protocols regardless of the PCR result. Despite its limitations, point-of-care PCRs have the potential to reduce diagnostic turnaround times and provide a potentially simpler option to caregivers; allowing for the screening high risk animals, reducing diagnostic guesswork, and ensuring well-timed enaction of biosecurity measures (Slovis *et al.*, 2020).

An alternative method to detect pathogen DNA directly that has shown promise in recent years are LAMP assays, able to amplify DNA at a constant temperature, requiring less specialist infrastructure and equipment than PCR (Parida *et al.*, 2008). LAMP assays have demonstrated functionality at the point of care by utilising a microfluidic device (Boyle *et al.*, 2021, Song *et al.*, 2016), albeit with a lower limit of detection than the point-of-care assay evaluated by Willis *et al.* (2021). A comparative study completed by Boyle *et al.* (2021) found that the LAMP assay, targeting *eqbE*, was reported to be comparatively sensitive and more efficient than the triplex assay (Webb *et al.*, 2013). However, the differing strains of *S. equi* prevalent in each region and design of the studies does not allow for fair comparison. Webb *et al.* (2013) analysed fresh samples animals with variable bacterial loads from the UK, whereas Boyle *et al.* (2021) analysed frozen samples from animals thought to have low bacterial loads from the USA. Comparative analysis would be required to ascertain if this LAMP assay (Boyle *et al.*, 2021) is preferable to quantitative PCR, ideally, with other gene targets, notably SEQ_2190, being incorporated.

PCR and LAMP assays detect DNA of live and dead bacteria indiscriminately; although this potentially results in false positive results as the number of viable organisms are overestimated, all positive cases should be taken seriously, even if they are also culture negative (Rendle *et al.*, 2021, Boyle *et al.*, 2018, Waller, 2014, Pusterla *et al.*, 2018). Efforts to determine the viability of *S. equi* using molecular approaches show promise (Pusterla *et al.*, 2018). By determining absolute quantitation using real-time quantitative PCR and

detecting mRNA transcripts such as those associated with virulence, insight into the physiological state of *S. equi* and therefore its viability can be revealed (Pusterla *et al.*, 2009, Pusterla *et al.*, 2005). In order to address the issue of poor transcript quality, the inclusion of a 24-hour culture step was investigated, although to no significant benefit in the determination of viability (Pusterla *et al.*, 2021).

Advances in diagnostics and surveillance are interlinked: techniques such as quantitative PCR for bacterial DNA (Webb *et al.*, 2013) and LAMP assays for specific *SeM* targets (McGlennon *et al.*, 2021) are rapid and possess high sensitivities and specificities. However, not all tests perform equally, and other factors such as sample site can affect the results (Lindahl *et al.*, 2013a). These technologies allow for the creation of clinically valuable surveillance schemes (McGlennon, 2019), with both laboratory and veterinary contributors. Point-of-care assays have limitations in detection threshold, but have the potential to reduce diagnostic turnaround times and provide a simpler option to caregivers (Slovis *et al.*, 2020). This would allow for the screening of high-risk animals, reducing diagnostic guesswork, and ensuring well-timed enaction of biosecurity measures.

The successful identification of *S. equi*, whether through bacterial culture or molecular methods, is dependent on the stage of infection (Rendle *et al.*, 2021) and the sampling site and technique used (Boyle *et al.*, 2017b). Using quantitative PCR, it was found that nasopharyngeal lavage was the optimal sampling technique for an acute *S. equi* infection with the highest sensitivity, followed by nasopharyngeal swabbing and then nasal swabbing (Lindahl *et al.*, 2013a). However, as noted by Rendle *et al.* (2021), the swabs used for this study are not ubiquitous globally and their surface area is smaller than those commonly used in the UK. Consequently, these results may not be conclusive or generalisable, although a nasopharyngeal wash examines a larger surface area than a typical swab, so would be expected to more frequently return *S. equi*. A single negative test result does not equate to the absence of infection and multiple different samples may be required to obtain a positive result (Boyle *et al.*, 2018). *S. equi* is only present transiently on the nasal mucosa and is often undetectable in a nasopharyngeal swab or wash sample until the lymphoid abscesses rupture, which typically occurs 1−4 weeks after infection (Rendle *et al.*, 2021). Similarly, guttural pouch washes will yield negative results in the initial stages of infection, until the retropharyngeal lymph nodes abscess rupture (Boyle *et al.*, 2018). Nasal swabs are

only recommended when an equid has active mucopurulent nasal discharge (Lindahl *et al.*, 2013a). Aspiration of a mature lymphoid abscess can be used to confirm *S. equi* infection and is often optimal during this stage of the disease (Boyle *et al.*, 2018).

1.4.2 Diagnosis of persistent *Streptococcus equi* infection

Carriers of *S. equi* do not differ clinically or on inflammatory markers, including WBC counts and SAA (Pringle *et al.*, 2020b). Carrier status therefore has little impact on systemic inflammation, and serology in isolation is insufficient to differentiate carriers and non-carriers (Christoffersen *et al.*, 2010, Davidson *et al.*, 2008). No method of diagnosis has the evidence to validate their use in screening for chronically infected animals, although guttural pouch endoscopy and lavage, with samples being tested by PCR and bacterial culture, is considered current best practice (Lindahl *et al.*, 2013a, Gronbaek *et al.*, 2006).

There is conflicting evidence on the utility of endoscopy scoring and *S. equi* has been demonstrated to produce a microscopic biofilm, microbiologically positive but grossly normal, with potential adhesive functions (Steward *et al.*, 2017). Many carriers have been shown to possess grossly normal guttural pouches at 6 months and beyond following an outbreak (Pringle *et al.*, 2020b, Riihimäki *et al.*, 2016). The timing of guttural pouch examination is likely to influence findings, as Boyle *et al.* (2017b) found distinct differences are visible in many carriers at a median time of three months after an outbreak.

Endoscopically guided guttural pouch lavage followed by quantitative PCR is recommended for the detection of persistent infections (Boyle *et al.*, 2018). This technique also allows for inspection of the guttural pouch, allowing identification of chondroids, inflammation, or empyema; although, contamination of equipment can result in false positive results (Svonni *et al.*, 2020). LAMP assays have been demonstrated to be comparable to PCR for this purpose (Boyle *et al.*, 2017b). Guttural pouch lavage has been validated as superior to a single nasopharyngeal swab or lavage (Boyle *et al.*, 2017b); however, nasopharyngeal lavage on three separate occasions has demonstrated to predict freedom from persistent infection (Pringle *et al.*, 2022a, Sweeney *et al.*, 2005). Pringle *et al.* (2022b) analysed a single outbreak, and it was advised that additional testing would be required to detect carriers as opposed to freedom from carrier status; ultimately, further comparative work is needed to

confirm their findings. Repeated nasopharyngeal lavage is also costly due to the need for recurrent veterinary action and risks incurring a high proportion of false negative results (Duffee *et al.*, 2015), although repeated testing mitigates this possibility. These potentially disparate studies (Pringle *et al.*, 2022a, Boyle *et al.*, 2017b, Pringle *et al.*, 2019) are not in conflict and can complement one another, resulting in more potential tools for detecting carriers. Guttural pouch lavage is invasive and costly, but repeated nasopharyngeal lavage requires additional call-out fees from veterinary professionals; as such, neither is obviously advantageous in terms of economics and practicality.

Serological testing is unreliable in identifying carrier animals (Durham and Kemp-Symonds, 2021, Davidson *et al.*, 2008, Pringle *et al.*, 2020b), and does not replace these other more invasive, expensive, and time-consuming methods of detection. Guttural pouch lavage combined with quantitative PCR is considered the best, albeit imperfect, method for carrier detection (Svonni *et al.*, 2020, Boyle *et al.*, 2018, Rendle *et al.*, 2021); although, economic and practical implications mean it is not always applicable.

1.4.3 Serological testing

Indirect enzyme-linked immunosorbent assays (iELISAs) detect antibodies generated by the host: in strangles they are used for screening animals (Craig, 2021), identifying exposure following an outbreak (Robinson *et al.*, 2013, Rendle *et al.*, 2021), and diagnosing the complications of strangles (Boyle *et al.*, 2009). Carrier status cannot be accurately determined using commercially available iELISAs (Durham and Kemp-Symonds, 2021, Van Maanen *et al.*, 2021). Commercially available iELISAs detect antibodies produced against the SeM surface protein, or both antigen A (SEQ_2190, a non-SeM target) and antigen C (a fragment of SeM) of *S. equi*, the so-called dual-target iELISA (Robinson *et al.*, 2013, Boyle *et al.*, 2018). Antibody titres decrease over time, and that antimicrobial therapy will result in a lower titre (Boyle *et al.*, 2017a, Sheoran *et al.*, 1997, Piche, 1984).

SeM-based iELISAs can be used to aid in the diagnosis of purpura haemorrhagica or metastatic abscessation (associated with titres ≥12,800), as well as identify animals predisposed to developing purpura haemorrhagica (titre >1:3,200) (Boyle *et al.*, 2018, Boyle *et al.*, 2009). They can also be used to indicate recent infection (≥4-fold increase in titre

between paired samples taken ten days apart) (Boyle *et al.*, 2009, Boyle *et al.*, 2018), although a single reading does not provide a measure of protection or active infection.

Cross-reactivity with a SeM homologue in *S. zooepidemicus* (Kelly *et al.*, 2006) combined with the failure of the SeM-based iELISA to detect *S. equi* strains not containing SeM (Harris *et al.*, 2015) led to the development of the dual-target ELISA (Duran and Goehring, 2021, Robinson *et al.*, 2013). The dual-target iELISA is reported to have similar sensitivity (93.3 vs 89.9%), but greater specificity (99.3 vs 77.0%) than the single target SeM-based iELISA (Robinson *et al.*, 2013).

Following an outbreak, it is advised to use the dual-target iELISA to identify horses exposed to *S. equi* (Boyle *et al.*, 2018, Duran and Goehring, 2021). The dual-target iELISA is reported to have similar sensitivity (93.3 vs 89.9%), but greater specificity (99.3 vs 77.0%) than the single target SeM-based ELISA (Robinson *et al.*, 2013). It can be used to identify recent exposure, from as little as two weeks post-infection, and has been used to determine exposure in populations across the globe (Ling *et al.*, 2011, Štritof *et al.*, 2021).

Retrospective analysis by Durham and Kemp-Symonds (2021) found no significant association between persistent infection and serological status. This retrospective study analysed a population of rescue animals which may not be representative of the wider equid population, and its design did not allow for confirmatory testing. The findings are contrary to much of the literature (Pringle *et al.*, 2020b, Knowles *et al.*, 2010) and casts doubt on current recommended guidelines (Boyle *et al.*, 2018). A recently developed avidity ELISA was reported to have no greater ability to detect persistent infections than ELISAs currently in practice (Van Maanen *et al.*, 2021). When screening for carrier status, serology is preferable to no diagnostic investigation (Rendle *et al.*, 2021); although, a false negative could result in a false sense of security. Serology remains a useful tool when assessing for exposure, but it is associated with high incidence of false negatives when samples are taken too early and false positives in vaccinated animals (Boyle *et al.*, 2018, Durham and Kemp-Symonds, 2021) which limits its utility, despite its prevalence in clinical practice.

1.5 Treatment of equine strangles

1.5.1 Treatment of acute *Streptococcus equi* infection

Most equids with acute strangles exhibit non-specific signs of generalised respiratory infection with presentation depending on challenge dose and host immunity, often responding well with only supportive and nursing care (Rendle *et al.*, 2021, Whitelegg and Saunders, 2021). In more severe cases, a hospital setting may be elected for, where more intensive treatment options such as intravenous fluid therapy (IVFT) and assisted nutrition can be offered. Acute disease can quickly deteriorate into severe cases thus the need for regular monitoring is emphasised (Rendle *et al.*, 2021).

Nursing for an animal with strangles is vital and wide-ranging: good nursing provision will include an environment that will encourage rest, appropriate nutrition, regular monitoring (TPR), abscess management, and a quarantine protocol (Whitelegg and Saunders, 2021). A soft, calorific, and palatable diet alongside water, to facilitate deglutition, both provided from a height, can help equids with profound lymphadenopathy; assisted nutrition may be indicated (Rendle *et al.*, 2021). The experience of individual equids must be considered during a strangles outbreak, as small changes in diet and environment can aid in assuaging the effects of infection with *S. equi*.

Individuals with visible lymphadenopathies, likewise, require good supportive and nursing care, the focus should be on facilitating the maturation and subsequent drainage of abscesses (Boyle *et al.*, 2018). The use of a 'hot pack' can enhance this process, alternative surgical drainage may be required if the abscesses are not spontaneously rupturing, although care must be taken to ensure the abscess is mature thus allowing maximal drainage (Boyle *et al.*, 2018). Once open, an initial lavage of abscesses with saline or antiseptic solutions should be followed by daily flushing so long as discharge persists (Rendle *et al.*, 2021).

Non-steroidal anti-inflammatories (NSAIDs) can be employed to provide analgesia and reduce pyrexia; it has been suggested that their use can slow the development of abscesses, but there is no evidence for this claim (Rendle *et al.*, 2021). Paracetamol is typically the drug of choice since it does not inhibit inflammation, but possesses anti-pyretic and analgesic

actions, resulting in improved appetite and welfare (Rendle *et al.*, 2021). Phenylbutazone or flunixin meglumine could also be considered (Boyle *et al.*, 2018).

Antimicrobial therapy has an important role in combatting *S. equi* infections but must be prescribed responsibly and only when clearly indicated, with careful consideration to minimise the development of antimicrobial resistance (Jaramillo-Morales *et al.*, 2022, Boyle *et al.*, 2018). For most strangles outbreaks, antimicrobials are not indicated or required for mature horses. Antimicrobials may be indicated between initial exposure and abscessation (Boyle *et al.*, 2018), but this window is not always adhered to since abscesses can develop within days (Timoney and Kumar, 2008). Although, antimicrobial therapy can decrease the size of abscesses and should be considered in equids with stridor, dyspnoea or dysphagia on welfare grounds, their effects are limited following the detection of lymphadenopathy (Boyle *et al.*, 2018). Systemic antimicrobials are indicated in animals following a tracheostomy, to prevent infection of the lower respiratory tract (Boyle *et al.*, 2018), although it is important to consider that the prognosis is poor in equids with associated laryngeal paralysis (Rendle *et al.*, 2021) presumably due to vagal nerve damage associated with the infection.

Penicillin is the drug of choice for *S. equi* infection; however, population analysis (Morris *et al.*, 2020) revealed that pbp2x mutations are emerging. This mutation is in the penicillin-binding site and is associated with penicillin resistance in *Streptococcus pneumoniae* (Maurer *et al.*, 2012, Nichol *et al.*, 2002). Although penicillin resistance has been observed in *S. equi* isolates (Fonseca *et al.*, 2020), it is not typically seen (Clark *et al.*, 2008, Johns and Adams, 2015) and further work is needed to determine the clinical implications of these conflicting findings. It is important that resistance is monitored, and that unusual results (Fonseca *et al.*, 2020) are followed-up in accordance with international standards (CLSI, 2020, EUCAST, 2023).

Overuse of antimicrobials can promote resistance (Jaramillo-Morales *et al.*, 2022) and lead to complacency (Boyle *et al.*, 2018). Reports on their use causing metastatic strangles are largely anecdotal (Ramey, 2007); indeed, even the prevalence of metastatic strangles is unclear (Spoormakers *et al.*, 2003, Sweeney *et al.*, 1987, Duffee *et al.*, 2015). The impact of antimicrobial use on the persistence of humoral immunity (Pringle *et al.*, 2020a) has been more rigorously demonstrated. There is a need for more clinical and experimental evidence

to explore the link between antimicrobial therapy and complications associated with *S. equi* infection.

1.5.2 Treatment of persistent *Streptococcus equi* infection

Persistent infections of the guttural pouch are typically treated with topical and prolonged systemic antimicrobial therapy (Boyle *et al.*, 2018): administration of penicillin systemically, and a gelatin-penicillin mix topically, endoscopically-guided, have been regarded as broadly successful (Verheyen *et al.*, 2000). The use of a reverse thermodynamic gel with benzylpenicillin presents an easier alternative to using a gelatin mix, where antimicrobial concentration is maximised since the gel can be retained in the guttural pouch for over 72 hours (Bowen, 2017, Rendle *et al.*, 2021).

The removal of purulent material and chondroids from the guttural pouches is required for the elimination of the carrier state (Boyle *et al.*, 2018). Endoscopic intervention is preferable to surgical intervention due to inherent risks of general anaesthesia, surgical dissection around vital structures, and *S. equi* environmental contamination (Boyle *et al.*, 2018). Topical application of 20% acetylcysteine (w/v) solution can facilitate drainage of non-inspissated mucopurulent material through the nasal passages by disrupting disulphide bonds, thereby reducing mucus viscosity (Boyle *et al.*, 2018). Described surgical approaches enter from either the lateral (Viborg's triangle, Garm's and hyovertebrotomy) or ventral (Whitehouse) aspect (Freeman and Hardy, 2018). It has been demonstrated that a modified Whitehouse approach can be performed, allowing the animal to remain standing in contrast to, reducing the risks of general anaesthesia and contamination (Perkins *et al.*, 2006, Schaaf *et al.*, 2006), but pharyngeal scarring could obstruct nasal drainage (Boyle *et al.*, 2018). Conversely, non-invasive methods can damage the guttural pouch lining, contaminate associated structures (Fogle *et al.*, 2007), and present practical challenges (Judy *et al.*, 1999).

1.5.3 Treatment of the complications of *Streptococcus equi* infection

Complications of strangles include metastatic strangles, where *S. equi* spreads to anatomical sites other than the regional lymph nodes, the type three hypersensitivity reaction purpura

haemorrhagica, and associated myopathies (Boyle, 2017). In all cases of complications associated with *S. equi* infection, supportive care is essential: ensuring adequate feed intake, closely monitoring for signs of deterioration, and providing an adequate environment are imperative to a successful recovery (Boyle *et al.*, 2018, Whitelegg and Saunders, 2021).

Metastatic strangles is treated with the drainage and sterile flushing of abscesses, whenever possible and dependent on their location (Boyle *et al.*, 2018). Ultimately, prolonged antimicrobial therapy, commonly penicillin, alongside good supportive care, is required for management and resolution (Berlin *et al.*, 2013, Pusterla *et al.*, 2007).

Purpura haemorrhagica is primarily treated with corticosteroids, typically dexamethasone at an anti-inflammatory dose (Pusterla *et al.*, 2003); if associated with an active bacterial infection, antimicrobial therapy is also indicated (Boyle *et al.*, 2018). NSAIDs and supportive therapy such as IVFT and hydrotherapy may also be of benefit in some cases (Boyle *et al.*, 2018).

Equids presenting with myopathies should be aggressively treated with corticosteroids; antimicrobial therapy, IVFT and physiotherapy may also be indicated (Boyle *et al.*, 2018). However, treatment is unsuccessful in many cases (Mallicote, 2015).

1.6 Prevention and management of equine strangles

1.6.1 Outbreak prevention

Strangles was once considered an inevitability (Solleysel, 1664), but has since been demonstrated to be a very preventable infection (Rendle *et al.*, 2021). Outbreaks can be prevented by limiting exposure to the infectious agent, through enacting rigorous biosecurity protocols, appropriate quarantining and screening facilities, and possessing an understanding of the pathogenesis of *S. equi* (Boyle *et al.*, 2018). Long term control strategies would also include the vaccination of unexposed animals, the identification and treatment of carrier animals, and caregiver education on clinical signs associated with acute disease (Duran and Goehring, 2021).

Additionally, *S. equi* has been isolated in face flies (*Musca autumnalis*); although, only 0.54% of flies tested positive for *S. equi* on qPCR and only a single outbreak was analysed (Pusterla *et al.*, 2020). Flies act as mechanical vectors in many diseases (Davies *et al.*, 2016, Chaiwong *et al.*, 2014) and may act as risk factor for strangles, highlighting the need for good biosecurity protocols and fly control despite the low level of detection (Pusterla *et al.*, 2020). Continuative work focusing on the link between fly control and *S. equi* infection has the potential to determine if flies are, indeed, a potential risk factor for strangles; although, equestrian facilities with rigorous biosecurity protocols are likely to have better fly control than facilities without such measures.

All new arrivals to farms should be quarantined for but at least 21 days, but optimally 28; if possible, they should be screened for subclinical infection by guttural pouch endoscopy, PCR, bacterial culture, and serology (Robinson *et al.*, 2013, Boyle *et al.*, 2018). Animals with high risk of exposure should be quarantined for 28 days (Rendle *et al.*, 2021). There are practical and financial implications for such rigorous protocols, indeed, ensuring owner compliance and appropriate control measures may be challenging (Rendle *et al.*, 2021), but identifying and treating carrier animals will reduce incidence of outbreaks. During breeding and competition seasons, interaction between equids is extensive and screening new arrivals is insufficient in isolation (Ikhuoso *et al.*, 2020).

Biosecurity measures are crucial to prevent outbreaks, including the disinfection of potentially contaminated equipment, avoidance of nose-to-nose contact between equids

not residing at the same farm, avoiding the sharing of feed and water buckets, and the disinfection of shared equipment (Sweeney *et al.*, 2005). Weese *et al.* (2009) reported that *S. equi* could survive on outdoor surfaces for up to four days, but that ultraviolet light in the form of intense sunlight resulted in the death of all bacteria. However, more recent analysis demonstrated that *S. equi* can persist in the environment for up to 34 days in cold, wet sites, such as shared water buckets (Durham *et al.*, 2018). These studies evaluated survival in different places, times, and temperatures; although the temporal ranges differed, they concur that *S. equi* can persist in the environment and can survive for longer in winter and in wet areas. Veterinary professionals must also enact strict biosecurity and disinfection protocols, as it was found that *S. equi* can survive inside a nasogastric tube for up to 21 days (Durham *et al.*, 2018).

1.6.2 Outbreak management

Outbreaks of strangles are controlled through the cessation of movement to and from the farm, isolating animals who are infected and where infection is suspected, and by creating a colour-coded tiered system of animals with no mixing between groups; following the outbreak all affected animals should be tested for carrier status (Boyle *et al.*, 2018).

A comprehensive history is first required: vaccination, travel and management history are vital (Boyle *et al.*, 2018). Using clinical presentations and thorough histories of all animals, the farm can be divided into three segregated colour-coded groups with red indicating clinical sign(s) consistent with *S. equi* infection, amber signifying direct or indirect contact with exposed animals, and green denoting unexposed animals (Boyle *et al.*, 2018, Duran and Goehring, 2021). Management of animals will vary on their grouping, with at least three meters of physical distance between them to avoid direct contact, and the regular monitoring of unaffected animals - rectal temperature should be measured and recorded twice daily (Boyle *et al.*, 2018). Equipment, and ideally staff, should be separate for each grouping to avoid cross contamination and spread via fomites (Durham *et al.*, 2018), if this is not possible then staff should work from the lowest risk group (green) to the highest (red) (Boyle *et al.*, 2018).

Other measures that should be considered when controlling an outbreak of strangles include the training of farm staff in basic biosecurity and nursing care, regular disinfection of water troughs, the exclusion of pets on the farm, suitable PPE provision, provision of hand washing facilities, regular disposal of bedding, and disinfection of contaminated areas (Rendle *et al.*, 2021).

Diagnosis should be conducted at the farm-level and not all animals with clinical signs will require laboratory confirmation, instead finances should be prioritised on testing for carrier status following the denouement of the outbreak (Rendle *et al.*, 2021). Testing for persistent infection should occur at least three weeks after clinical signs cease or antimicrobial therapy has concluded, with animals designated non-carriers on the basis of no gross guttural pouch pathology, as well as a negative qPCR result (Boyle *et al.*, 2018). Performing three nasopharyngeal lavages on separate occasions, subject to PCR analysis, have also been used to demonstrate freedom from carrier status (Pringle *et al.*, 2022b). Owner compliance and financial implications often determine which method is used in practice; although, both methods are ultimately costly and time-consuming (Boyle *et al.*, 2018, Pringle *et al.*, 2022b).

Effective use of human behaviour science, along with clear and concise messaging (Rendle *et al.*, 2021), can support veterinary professionals in dealing with farm managers and animal caregivers, resulting in improved compliance notably in the implementation of biosecurity measures (White *et al.*, 2021). Designating a leadership structure to an outbreak, especially if multiple veterinary practices are involved, providing caregivers with a point of contact, and involving those affected in the decision-making process in deciding biosecurity protocols, treatment strategies, and exit strategies can aid in this goal (Rendle *et al.*, 2021). Veterinary teams must be realistic about financial and temporal implications and provide an opportunity for questions to be answered; ultimately, the changes being implemented must be 'owned' by caregivers (White *et al.*, 2021).

At a 2019 Strangles symposium, the Human Behaviour Change for Animals (HCBA) facilitated a workshop of stakeholders, focusing on strangles prevention and management (White, 2019). From this symposium, the model of transtheoretical change (Prochaska and Velicer, 1997), visualising change as process with stages that must be moved through, and the COM-B behaviour change wheel (Michie *et al.*, 2011), focusing on a person's capability, opportunity, and motivation to enact a behaviour, were deemed to be relevant to the

control of strangles. Adopting models of human behaviour can have the ability, at the national, farm and individual level, to address caregiver compliance (White *et al.*, 2021) and create positive messaging around vaccination (Rendle *et al.*, 2021), with the potential to reduce the spread of *S. equi*.

1.6.3 Vaccination

The ideal strangles vaccine should provide a high degree of protection against *S. equi*, a long duration of immunity, the ability to be administered intramuscularly safely, and permit the differentiation of infected from vaccinated animals (DIVA) (Waller and Jolley, 2007). DIVA capability is important since the current commercially available enzyme-linked immunosorbent assays (ELISAs) do not differentiate between recently exposed horses and those animals vaccinated with live-attenuated vaccines, with implications for screening animals, movement restrictions and disease control (Duran and Goehring, 2021).

The first strangles vaccines were developed in the 1940s, using heat-killed bacteria, conferring a limited degree of protection, but often resulting in adverse effects including injection site reactions and pyrexia (Bazeley, 1940a, Bazeley, 1940b, Bazeley, 1942a, Bazeley, 1942b, Bazeley, 1943). Cell-free variations of this vaccine still exist (Waller, 2014), although the prevalence of adverse reactions and the lack of DIVA capability has limited their use. A recent attempt to combine the *S. equi* bacterin and recombinant SeM protein in a vaccine yielded promising results in mice with all demonstrating a humoral response (Rosa *et al.*, 2021); evaluation of its safety and efficacy in horses is ongoing.

M-protein-containing extract vaccines have demonstrated some efficacy in reducing the frequency and severity of disease; although adverse reactions are common and they possess no DIVA capability (Hoffman *et al.*, 1991). In a double-blind randomised clinical trial in foals, 29% (17/59) of vaccinates developed cervical lymphadenopathy, compared to 71% (39/55) of sham-vaccinated controls (Hoffman *et al.*, 1991). Commercially available options, although none are available in the UK, include Strepvax II (Boehringer Ingelheim), Equivac S (Zoetis New Zealand), and Strepguard (MSD Animal Health) (Duran and Goehring, 2021).

Live-attenuated vaccines have been at the forefront of strangles prevention since the early 21st century; a 10^{9} dose of an avirulent strain of *S. equi*, was shown to prevent

lymphadenopathy in 100% (5/5) and 50% (2/4) of ponies, respectively, across two experiments conducted by Jacobs *et al.* (2000). The Equilis StrepE (MSD Animal Health) is administered submucosally, and the Pinnacle IN (Zoetis) is administered intranasally; they are commercially available in Europe and North America, respectively, as well as other countries intermittently (Duran and Goehring, 2021). Adverse reactions were reported upon intramuscular administration, and these live-attenuated vaccines possess no DIVA capability (Kemp-Symonds *et al.*, 2007, Borst *et al.*, 2011, Livengood *et al.*, 2016, Lanka *et al.*, 2010). Furthermore, the Equilis StrepE (MSD Animal Health) vaccine has been linked to *S. equi* replication, resulting in lymph node abscesses (Kemp-Symonds *et al.*, 2007, Kelly *et al.*, 2006, Mitchell *et al.*, 2021, Harris *et al.*, 2015).

Strangvac (Intervacc AB) is a recombinant fusion protein vaccine that is administered intramuscularly and has been shown to protect up to 94% (15 of 16) ponies from clinical signs of disease, including the development of abscesses in the retropharyngeal or submandibular lymph nodes when challenged two weeks following third vaccination (Robinson *et al.*, 2020). Strangvac has DIVA capability as the vaccine does not contain live *S. equi*, S*. equi* DNA nor the *SeM* and SEQ_2190 antigens that are targeted by culture, PCR, or ELISA diagnostic tests (Robinson *et al.*, 2018). Future studies will be needed to evaluate the utility of Strangvac (Intervacc AB) in clinical practice.

Vaccination as a tool for outbreak prevention has been limited by efficacy, safety, practicality, clashes with other vaccination schedules, DIVA capability, geographical restrictions, differences in circulating *S. equi* strains and owner compliance (Boyle *et al.*, 2018, Mitchell *et al.*, 2021). Advancements such as the Strangvac vaccine represent a promising development, potentially allowing vaccination to become a more efficacious control measure. However, continued work is required from veterinary professionals to build trust with owners and caregivers over the use of any strangles vaccines due to past difficulties (White *et al.*, 2021).

1.7 *Streptococcus equi*

1.7.1 Evolution of *Streptococcus equi*

S. equi and *S. zooepidemicus* are closely related, sharing over 97% of their DNA (Jorm *et al.*, 1994, Webb *et al.*, 2008). There is a growing body of evidence to suggest that *S. equi* evolved from ancestral *S. zooepidemicus*, after passing through a genetic bottleneck, resulting in its eventual host-restriction through a process of simultaneous gene loss and gain (Holden *et al.*, 2009, Harris *et al.*, 2015, Webb *et al.*, 2008). The deletion of the clustered regularly interspaced short palindromic repeats (CRISPR) locus in *S. equi* is thought to have favoured the procurement of novel genetic elements, albeit at the expense of genome stability (Waller and Robinson, 2013).

S. equi, *S. zooepidemicus*, and the human pathogen *Streptococcus pyogenes* (*S. pyogenes*) share a common phage pool that has enhanced their cross-species evolution, with their individual developments being a result of functional loss, pathogenic adaptation, and genetic exchange (Holden *et al.*, 2009). *S. equi* and *S. zooepidemicus* have functional differences in carbohydrate metabolism (Bannister *et al.*, 1985), hyaluronic acid synthesis (Holden *et al.*, 2009), and cell wall-anchored proteins (Lindmark *et al.*, 2001), as *S. equi* has refined its requirements and capabilities similar to other host-restricted pathogens (Parkhill *et al.*, 2003).

1.7.2 Genome of *Streptococcus equi*

The *S. equi* genome is larger than that of *S. zooepidemicus*, in-part because of its plasticity and the procurement of many mobile genetic elements (MGEs), crucial in the evolution of *S. equi* as a pathogen (Holden *et al.*, 2009). Another important difference between the two genomes is the notable presence of the gene ICESe2, incorporating the entirety of the equibactin locus involved in iron acquisition (Heather *et al.*, 2008). This species difference is indicative of how novel functions were introduced, at the expense of ancestral capabilities (Holden *et al.*, 2009). The loss of genes not required to cause disease may have streamlined the *S. equi* genome, at the expense of becoming host-restricted and only being able to cause disease in equids (Waller, 2016).

The genome of *S. equi* is largely homogenous, with few differences between isolates found in housekeeping genes when analysed by multi-locus sequence typing (MLST) or multi-locus enzyme electrophoresis (MLEE) (Webb *et al.*, 2008, Jorm *et al.*, 1994). The SeM protein is a surface antigen of *S. equi*, with a function similar to that of the M protein of *S. pyogenes*: by binding to fibrinogen and IgG, C3b complement deposition is inhibited, thus phagocytosis can be resisted (Timoney *et al.*, 1997, Meehan *et al.*, 2000, Meehan *et al.*, 2009). SeM is not present in *S. zooepidemicus*, although a homologue exists, and it is considered a contributing factor to the greater virulence of *S. equi*. The 5' N-terminal region of the *SeM* gene is hypervariable, even within a local population of equids, and therefore can be analysed to discriminate between isolates (Parkinson *et al.*, 2011) and identify the sources of outbreaks (Kelly *et al.*, 2006). The hypervariability of this N-terminus does not affect fibrinogen-binding but can interfere with antibody (IgA but not IgG) and T cell-mediated immunity (Timoney *et al.*, 2010). The truncation of the N-terminal region of *SeM* is thought to contribute to immune evasion, allowing *S. equi* to persist undetected in the host (Chanter *et al.*, 2000), although evidence on this is not conclusive with isolates causing persistent disease being found with untruncated N-terminal regions in outbreaks in the USA and Sweden (Morris *et al.*, 2021)

Superantigens are toxins with the potential to initiate inappropriate and non-specific T-cell and cytokine production (Fraser *et al.*, 2000). Superantigens are not processed by antigen presenting cells (APCs) as smaller antigens are, they instead bind to major histocompatibility complex (MHC) class II molecules directly, in high concentrations T-cell receptors of T cells bind to the superantigen resulting in its activation and subsequent overzealous proliferation as well as cytokine production (Fraser and Proft, 2008). Associated with the acquisition of four prophage-encoded genes, *S. equi* produces four superantigens (*SeeH, SeeI, SeeL, SeeM*) which play crucial roles in its pathogenicity through the stimulation of an inappropriate and excessive Th1 response, potentially interfering in the establishment of an efficient and efficacious immune response (Paillot *et al.*, 2010b, Alber *et al.*, 2005). Their presence was variably noted in a studied Strangles outbreak in Egypt, with no difference in clinical presentation in isolates missing one or two superantigens (Tartor *et al.*, 2020).

1.7.3 Genetic epidemiology of *Streptococcus equi*

Equids are widely used and transported between geographic regions and strangles continues to spread as rapidly as ever (Mitchell *et al.*, 2021, Leadon *et al.*, 2008). Carrier animals with no outward clinical signs present a major barrier to preventing the spread of strangles. It is also difficult to assess the number and location of equids, infected or otherwise, due to limited controls on equid ownerships and transport (Robin *et al.*, 2011).

Population analysis of 670 isolates from 19 countries (Mitchell *et al.*, 2021) revealed the extent of the international transmission that results in the endemicity of strangles across the world. *SeM* was found to be a region of variability, with 103 different alleles being within the collection. Bayesian analysis and phylogenetic reconstruction revealed six clusters of genetically related sequence types (BAPS-1 – BAPS-6). BAPS-1 – BAPS-4 were dominant in different geographical regions, although BAPS-1 – BAPS-3 were present elsewhere; BAP-5 was revealed to be declining in prevalence and BAP-6 was found comparatively less frequently. Regions with relatively isolated trading patterns were clustered together, such as isolates from Oceania in BAPS-3, whereas BAPS-1, predominant in the USA, was linked to outbreaks in Israel, Japan, and the UAE.

1.7.4 Genomic changes in chronically infective isolates

S. equi has been characterised as possessing a dynamic genome, with the ability to diversify and decay; mutations relating to metabolic streamlining and the loss of virulence has been noted in chronically infective isolates (Harris *et al.*, 2015). The endemicity of *S. equi* can, in-part, be attributed to its ability to persist in the guttural pouch following an infection, surviving in a low-nutrient state yet intermittently shedding bacteria and thus exposed naïve animals.

Genomic decay during persistent infection may reduce transmissibility and result in a lessened ability to cause severe acute disease, although the organism undoubtedly remains infectious, indeed this is a key step in the disease cycle (Harris *et al.*, 2015). Individuals with residual immunity, such as equids that are older or vaccinated, and foals with maternal antibodies can develop a milder form of disease, termed ‘atypical strangles’ (Prescott *et al.*, 1982). This presentation may be caused by a reduction in virulence in isolates where

deletions in the genome are present (Waller, 2016), as with the outbreak described by Tscheschlok *et al.* (2018) where the strain of *S. equi* had a deletion in the SEQ_0402 gene, which likely attenuated it.

Persistent bacteria must resist an effective immune response in an altered environment, with different genes being required to become long-term residents than are needed to cause acute disease (Waller, 2016). Population analysis has suggested that persistent infection reduces pathogenic potential of *S. equi*, with long-term resident isolates found to be less virulent and more metabolically streamlined (Harris *et al.*, 2015). This genomic diversification and decay results in adaption to the guttural pouch environment, including the loss of genes involved in citrate metabolism, the hyaluronic acid capsule, and the equibactin siderophore. The loss of genes involved in hyaluronic acid synthesis mirrors the pathogenesis of *S. pyogenes*, allowing *S. equi* infection to be used as a model for human disease (Flores *et al.*, 2012, Shea *et al.*, 2011).

Analysis by Morris *et al.* (2021) of the genome, methylome, and transcriptome of *S. equi* isolates found no significant differences between isolates causing acute disease and those causing persistent infection, this was also the case in accessory genome elements, although inconsistent minor differences were detected. Genomic elements associated with carrier states were variably found in persistent isolates: truncation of the N-terminus of *SeM* was not consistently found and no isolates were found to have lost the equibactin locus. Whilst no consistent changes in the genomes of isolates from carrier horses were identified, the genomes of some individual isolates recovered from persistently infected horses were found to contain deletions in *SeM* and the citrate locus. Therefore, Morris *et al.* (2021) arguably provides further evidence of genome decay in isolates from persistently infected horses that differs from one carrier to another, potentially reflecting the varied selective pressures that are exerted on *S. equi* as this organism persists within the guttural pouch.

Inconsistent results (Harris *et al.*, 2015, Morris *et al.*, 2021) may be indicative of the differing methodologies employed or due to the constraints of the data analysed. Harris *et al.* (2015) employed Illumina sequencing to analyse 224 isolates from multiple sources: 180 from outbreaks across the UK, 40 geographically diverse isolates to provide a global sample, and isolates from commercially available vaccines. In contrast, Morris *et al.* (2021) used PacBio and Illumina (NovaSeq 6000) sequencing to respectively analyse the genome and

transcriptome of a smaller sample of 35 isolates: 14 from a single outbreak in Sweden and 21 from multiple outbreaks across Pennsylvania, USA. Although data from 2 geographically distinct regions were included, the sample size is smaller and from fewer outbreaks than the work presented by Harris *et al.* (2015), thus it not clear that this population analysis can be extrapolated to the global *S. equi* population. The variability in strains, notably those of different geographical and temporal origin, is also confounded across studies (Morris *et al.*, 2021, Harris *et al.*, 2015, Morris *et al.*, 2020), contributing to the intricate picture presented.

A complex interplay between the host and causative agent is suggested in which genomic plasticity could play a central role; this is an opportunity for further research with an emphasis on understanding host, as well as pathogenic, factors such as immunity.

1.8 *Streptococcus zooepidemicus*

1.8.1 *Streptococcus zooepidemicus* as a disease-causing agent

S. zooepidemicus is a diverse subspecies that has the potential to cause a variety of infections in a wide range of mammalian hosts, commonly causing respiratory or reproductive disease in equids and ruminants (Waller, 2010, Li *et al.*, 2021), as well as in dogs (Priestnall *et al.*, 2010), rodents (Riggs, 2009), swine and primates (Salasia *et al.*, 2004). *S. zooepidemicus* is a zoonotic pathogen; although infection in humans is rare, it has the potential to cause severe disease (Pelkonen *et al.*, 2013, Beres *et al.*, 2008). Infection in equids is often linked to immunocompromise, such as during viral infection, or stress as a result of travel, injury or heat (Timoney, 2004).

S. zooepidemicus is commonly isolated from the respiratory mucosa of healthy and diseased equids alike (Pansani *et al.*, 2016, Zhu *et al.*, 2021); consequently, debate exists over whether it is a commensal, primary pathogen, or an opportunistic pathogen, with consensus shifting towards the latter (Javed *et al.*, 2016, Waller, 2017, Timoney, 2004). Strains of *S. zooepidemicus* have been shown to cause outbreaks across the globe with notable examples in Iceland (Björnsdóttir *et al.*, 2017), Sweden (Lindahl *et al.*, 2013b), the UK (Lindahl *et al.*, 2013b), and Ethiopia (Laing *et al.*, 2021). As a result, some are beginning to consider *S. zooepidemicus* as a primary pathogen (Laing *et al.*, 2021).

It has been postulated that historic low resolution sequencing methods, unable to differentiate between *S. zooepidemicus* strains, led to the assumption that this organism is a commensal (Waller and Wilson, 2021). As high resolution sequencing techniques, such as MLST, have become available, virulent strains have been identified (Waller and Wilson, 2021), as well as strains adapted to specific species and tissues including the equine respiratory tract (Velineni and Timoney, 2013).

When diagnosing *S. equi* infection, the possibility of *S. zooepidemicus* infection should also be considered, since its role as a primary pathogen needs to be further elucidated. However, treatment and medical management of non-strangles lymphoid abscessation is the very similar that of strangles (Rendle *et al.*, 2021).

1.8.2 Genome of *Streptococcus zooepidemicus*

As discussed, *S. equi* is believed to have evolved from an ancestral *S. zooepidemicus* leading to its host-restriction (Holden *et al.*, 2009); yet, over 97% of DNA is homologous between the two subspecies (Jorm *et al.*, 1994, Webb *et al.*, 2008). This genomic similarity highlights their intimate relationship; therefore, it is unsurprising that strains of *S. zooepidemicus* have the potential to cause acute disease, similar to *S. equi*. Indeed, it has previously been demonstrated that genetic exchange of MGEs occurs between *S. equi*, *S. pyogenes*, and *S. zooepidemicus* (Holden *et al.*, 2009).

There is evidence to suggest that animals build up immunity to *S. zooepidemicus* infections through continued exposure (Newton *et al.*, 2008, Wood *et al.*, 2005), implying pathogenicity. Genomic evidence of the ability of *S. zooepidemicus* to establish an infection includes the presence of *SzM* (Velineni and Timoney, 2013), *SzPSe* (Timoney *et al.*, 1997), *SclC* (Karlström *et al.*, 2004), *CNE* (Lannergard *et al.*, 2003) and other cell-surface proteins: these can play roles in immune evasion, cleavage of immune-activation factors such as IL-8 (Turner *et al.*, 2009) as well as adhesion and attachment (Lindmark *et al.*, 2001, Waller, 2017).

S. zooepidemicus, similar to many other streptococcal pathogens including *S. pyogenes* (Rohde and Cleary, 2016), has pili on its surface (Skive *et al.*, 2017) aiding with adherence and, ultimately, survival (Tsai *et al.*, 2017, Becherelli *et al.*, 2012). Superantigens, such as *SzeF*, *SzeN* and *SzeP*, have also been variably found across isolates, with their presence being significantly associated with lymph node abscessation (Rash *et al.*, 2014, Paillot *et al.*, 2010a), although *SzeF* was found to be functionally redundant.

These genomic elements allow for the establishment and maintenance of an infection, but are targeted by the host immune system, necessitating a fine balancing act (Waller, 2017). Additionally, recombinant vaccines can target many of these cell surface proteins, as is the case with the Strangvac vaccine (Intervacc AB) and *S. equi* (Guss *et al.*, 2009, Robinson *et al.*, 2018). If *S. zooepidemicus* can survive a targeted immune response, it may lead to a persistent infection and intermittent shedding, infecting unexposed animals similar to the evolutionary model followed by *S. equi* (Waller, 2017).

1.8.3 Phase variation

Phase variation is an example of reversible phenotypic variation. This variation can manifest in different ways, including changes in colony opacity, encapsulation, and pilus expression, allowing bacteria to adapt to different environmental conditions and stressors (Kwun et al., 2023). In Streptococcal species such as *Streptococcus Pneumoniae*, phase variation is driven by reversible switches of the methylomes or methylation patterns of the bacterial genome (Li and Zhang, 2019).

The surface architecture of *S. zooepidemicus* dictates the range of environments this opportunistic pathogen can persist in, and modulation in this way could assist in its varied lifestyle. *S. zooepidemicus* has been observed persisting through a mature immune response (Lindahl *et al.*, 2013b), this could in-part be due to inverting DNA sequences that represent an binary phenotype switch (Steward *et al.*, 2015).

PinR, a serine recombinase encoded by the gene SZO_08550, controls the production of the region upstream from SZO_08560; in turn, this determines the transcription of a surface protein with mucin-binding properties (Steward *et al.*, 2015). Expression of such a protein may be advantageous, depending on the environment, allowing for adherence (Ebbes *et al.*, 2011); however, it may act as a pathogen-associated molecular pattern (PAMP). Phase variation is, likewise, exhibited at the FimIV locus, again, mediated by PinR with mutants lacking PinR being unable to invert this region (Steward *et al.*, 2015).

Biphasic variation has also been observed in the devastating pathogen of swine, *Streptococcus suis (S. suis)*, where it was demonstrated that phase-variable methyltransferases are segregated across clades (Atack *et al.*, 2018). Phase variation has complicated the development of vaccines and therapeutics targeted against this pathogen. Further research is required to determine if this phenomenon is distinctly defined by genetic lineage in *S. zooepidemicus*, and whether biphasic variation is present in persistent isolates *S. equi*.

2 The screening of strangles: an evaluation of the strangles-screening process in a UK rescue centre

2.1 Background

Equine strangles is an ancient and highly infectious disease caused by *Streptococcus equi* subspecies *equi* (*S. equi*) that has significant implications for the health and welfare of equids across the globe. The continued persistence of equine strangles can largely be attributed to the presence of carrier animals, in which a viable population of *S. equi* persists and continues to shed, intermittently or continuously, following the apparent resolution of infection.

About 10% of equids enter the carrier state following acute infection, although this figure is highly variable between outbreaks and may be an underestimate. By the 20th century, risk factors for persistent *S. equi* infection had been described (Todd, 1910), including age and body condition; however, no host factors are consistently associated with infection (Ling *et al.*, 2011, Duffee *et al.*, 2015). Management-related factors, such as admission policy and biosecurity (e.g., the sharing of water buckets) within an equestrian facility, are the only factors consistently associated with outbreaks (Libardoni *et al.*, 2016, Laing *et al.*, 2021).

The identification and treatment of persistently infected equids is an important factor in the control of strangles and the prevention of outbreaks; this could be done in the aftermath of an outbreak or as a dedicated screening protocol to an equestrian facility. The implementation of pragmatic biosecurity measures such as good fly control (Pusterla *et al.*, 2020), disinfection of contaminated equipment, and avoidance of sharing feed and water buckets are crucial in preventing the environmental persistence of *S. equi* (Durham *et al.*, 2018). Effective long-term control strategies should also encompass the vaccination of unexposed animals.

There are many challenges associated with detecting carrier animals and there can be confusion around the effectiveness of the options available to caregivers and clinicians. Guttural pouch endoscopy and lavage is considered the best method of carrier detection, as it allows for a visual inspection of the guttural pouch alongside microbial analysis (Boyle *et al.*, 2018). Repeated nasopharyngeal lavage, on three occasions at least two weeks apart,

has been shown to predict freedom from persistent *S. equi* infection (Pringle *et al.*, 2022b), but this is not equivalent to reliably identifying equids that are persistently infected. Repeated nasopharyngeal lavage is also costly due to the need for recurrent veterinary action and risks incurring a high proportion of false negative results (Duffee *et al.*, 2015); although, repeated testing mitigates this possibility. The dual target iELISA is unable to reliably identify carrier animals and its main use is in screening for exposure to *S. equi* following an outbreak. All methods of carrier detection have drawbacks and there is not yet the evidence to validate any; however, guttural pouch lavage is recommended as the most effective method of diagnosing persistent *S. equi* infection, if economic and practical factors allow.

The aim of this study was to evaluate the effectiveness of a strangles screening protocol at a UK welfare centre equids (Bransby Horses, UK) including investigating the efficacy of the current screening tests and identifying host risk factors associated with strangles carriage.

2.2 Methods

2.2.1 Study design

In this retrospective cross-sectional study, the strangles screening data at a UK rescue centre was evaluated through the extraction and analysis of clinical records of all admitted horses between 2017 and 2021 (03Jan2017 – 14Dec2021).

To retrospectively confirm the strangles-free status of the herd, records for all cases of respiratory disease between 2019 and 2021 were reviewed, extracted, and analysed. This was done by using monthly key performance indicators (KPI) provided by Bransby Horses, and by evaluating the daily clinical diary between 2019 and 2021; from there the clinical records of all equids with respiratory disease or respiratory signs were analysed and recorded.

2.2.2 Setting

Bransby Horses UK is one of the UK's largest equine welfare charities comprising a primary centre with over 300 equids and a subsidiary site with around 50 equids. In addition, around 500 horses are fostered out at any one time. Bransby Horses UK established a strangles screening protocol following a severe strangles outbreak in 2008 that resulted in significant morbidity and mortality. The charity has a dedicated admission unit which all horses pass through upon arrival for veterinary assessment and strangles testing. This admission unit acts as a quarantine facility where equids pass through, on a rolling (i.e., not all-in/all-out) basis, for a minimum of 6 weeks. Once equids enter the general herd, all animals are checked at least once daily by a trained member of staff.

2.2.3 Participants

The target population was identified as equids admitted to the Bransby Horses welfare centre between 2017 and 2021 (03/01/2017 – 14/12/2021) that were screened for strangles. Equids that were admitted but not tested for strangles, such as those that were euthanised shortly after arrival, were not included in this study. Equids that were admitted more than once during this timeframe, for example animals that had been to hospital or

fostered out and returned, were treated as a separate case for each time they went through the screening process.

The screening protocol included a clinical examination, paired serology samples (iELISA) taken six weeks apart, and endoscopically guided guttural pouch lavage of both guttural pouches which were sent for quantitative PCR and culture analysis at a veterinary laboratory (Rainbow Equine Hospital, UK). Equids that were subject to at least one aspect of this screening protocol were included in this study, for example foals that were tested serologically but were too small to have endoscopy performed. For the duration of the strangles screening process (approximately six weeks), equids were housed in a dedicated quarantine unit, under the care of an onsite veterinary team.

2.2.4 Variables

A bespoke data capture form was created to collate the clinical data. Numeric identification codes were assigned to the admitted equids to provide anonymity and denote chronology. The bespoke data capture form was separated into three sections, contextual data, strangles-screening data, and blood test data. These are described below:

Contextual data contained information on key dates such as date of admittance and sampling and clinical examination findings. Age, breed, body condition score, sex, and neuter status of equids were also recorded in this section, where available.

Strangles-screening data contained results from the specific tests performed as part of the strangles-screening process for admitted equids (serology, endoscopy, and guttural pouch lavage), as well as a description of relevant clinical signs or guttural pouch abnormalities, such as lymphoid hyperplasia or the presence of chondroids. Fluid from guttural pouch lavage was sent for quantitative PCR and microbial analysis; the results from these tests were designated either ‘positive’ or ‘negative’. Cycle threshold values were not available for many equids that were PCR positive, so the decision was made to not record these values. The raw optical density (OD) data from the serological test, a dual target iELISA, were initially recorded. These data were later converted to a ‘positive’ (OD ≥ 0.5), ‘negative’ (OD < 0.3), or ‘equivocal’ (OD ≥ 0.3 < 0.5) result to allow for analysis.

Blood testing data included results from biochemistry and haematology tests on samples, typically taken during the initial veterinary assessment and at time of endoscopy. The raw data from these blood tests were initially recorded, and these results were later converted to 'elevated', 'normal', or 'lowered', as defined by the laboratory's reference range for each parameter (Rainbow Equine Hospital, UK).

Once recorded, data were cleaned in a systematic manner to allow for the identification of discrepancies and inputting errors, ensuring validity. Free text data and continuous data were sorted into discrete categories where possible, such as equid breeds (Table 2.1).

Table 2.1: Categorisation of breeds/types of equid as described in the clinical records of equids admitted to a UK rescue centre.

Classification	Breed or Type
Donkey type	Donkey, Mule
Small pony	Shetland (X), Welsh Section A, Miniature horse, Miniature Shetland, Falabella
Large pony	Pony, Welsh Section B, C, Welsh (X), Fell, Connemara (X), Riding pony, Highland (X), New Forest (X), Dartmoor, Dales, Haflinger
Arab type	Arab (X), Anglo Arab
Cob type	Cob (X), Welsh Section D, Welsh Cob
Light horse	Thoroughbred (X), Morgan, Appaloosa, Hackney (X), Trotter, Standardbred, Riding Hack
Sports horse type	Warmblood (X), Irish Sport Horse, Andalusian, Lipizzaner, Oldenburg, French, Fresian, Irish Draught (X), Irish (X), Westphalian, Selle Française, Holstein, Hanoverian (X), Gelderlander, Cleveland Bay (X), Trakehner
Other	Shire (X), Clydesdale (X), Percheron (X), unknown/unspecififed

'X' = crossed with another breed/type

2.2.5 Sources and handling of data

Using the IDEXX Animana system, admitted equids were ranked by date of first admission. Each animal's clinical records could then be accessed to allow for data retrieval. When clinical data on admitted equids could not be retrieved in this way, for example if they had been readmitted or were deceased, a copy of the paper list of admitted animals was

obtained from Bransby Horses. The clinical records of equids that did not appear on the electronic admittance list could then be individually searched for and retrieved.

2.2.6 Statistical methods

Descriptive statistical analysis was used to examine demographic and contextual data, with range, mean, median and mode being used to examine continuous variables and percentage frequencies calculated for categorical data. A similar methodology was employed to evaluate the strangles-screening and blood testing data, both of which were later analysed using two-way pivot tables in Microsoft Excel v2211 to examine the interactions between groups. The statistical software package GraphPad Prizm v9.4.1 was used to conduct further analysis and perform statistical analysis including Fisher's exact and Chi-squared tests to investigate the association between strangles and host risk factors, blood results and seropositivity.

2.2.7 Limitations and bias

This study is a retrospective clinical study and, as such, the laboratory results could not be verified at a second laboratory. Potential reasons for failure of laboratory tests, such as the ELISA serological test, are discussed in this study. In addition, cycle threshold values for positive PCR results were not available for many of the equids due to the retrospective nature of this study.

2.3 Results

2.3.1 Participants

The clinical records of 662 equids were extracted for the five-year study period. After equids that had not been tested for strangles, serologically or endoscopically, had been removed, the clinical records of 626 equids remained. Not all elements of the screening protocol were applied to all admitted equids, and in some cases the clinical data were not recorded or could not be retrieved. This is broken down by variable in Table 2.2, and described in the results as n=x/y.

Table 2.2: Population demographics and clinical data retrieved from a population of equids admitted to a UK rescue centre

Variable	Recorded	Not recorded or unable to retrieve data
Age	619	7
Breed	617	9
Sex	624	2
Body condition score	571	55
Endoscopy and guttural pouch lavage	561	65
Serology at initial vetting in	622	4
Serology at time of endoscopy (approximately 6 weeks after admission)	531	95
Biochemistry and haematology	360	266

2.3.2 Demographics of admitted equids

There were 626 equids admitted to Bransby Horses UK between 2017 and 2021 that were tested for strangles (Figure 2.1). Notably fewer equids were admitted during 2020 (n=82/626) compared to other years (n=126/626, 133/626 and 144/626 in 2017, 2018 and 2019, respectively, and n=141/626 in 2021) with the first three quarters of 2020 having the lowest admission numbers across the study timeframe. There was no discernible seasonal variation in admission numbers although the highest numbers of admitted equids were recorded in the first quarter of 2019 (n=53/626) and 2021 (n=52/626).

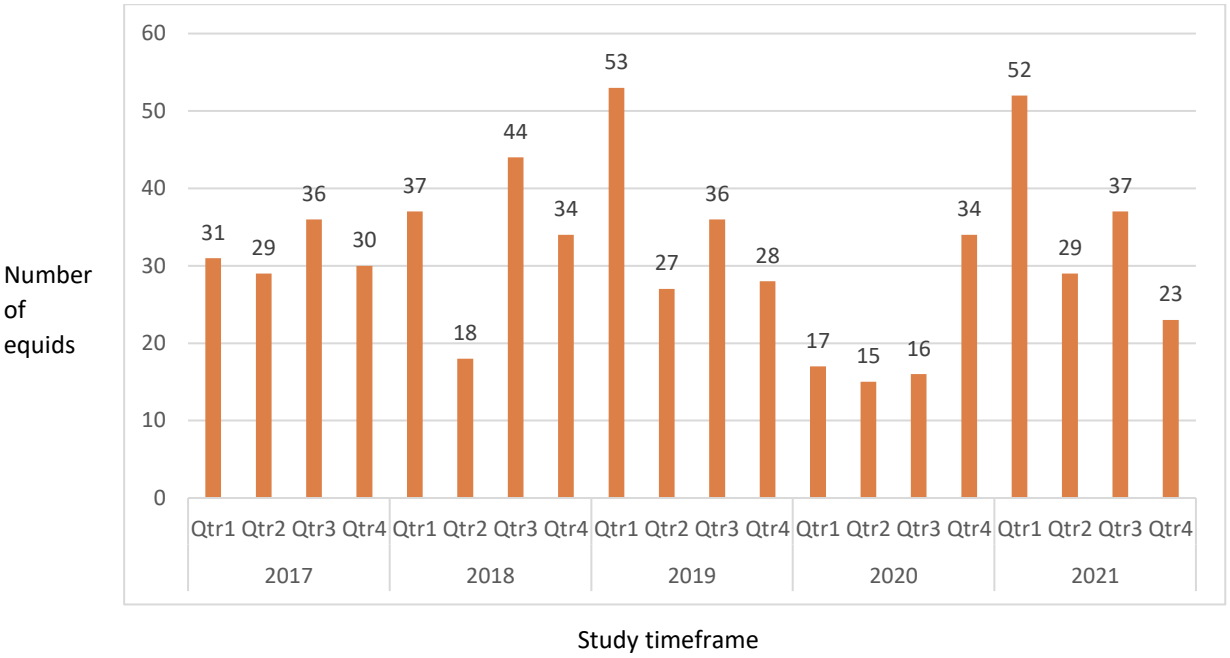


Figure 2.1: The time course (quarter/year) distribution of 626 equids that were tested for strangles in a retrospective study of equids admitted to a UK rescue centre between 2017 and 2021.

The majority, 60.6%, of equids admitted (n=375/619), were less than 10 years old and only 9.9% (n=61/619) of admitted equids were over 20 years old (Figure 2.2). The modal age was between five and nine years old with 32.3% (n=200/619) of admitted equids being in this category.

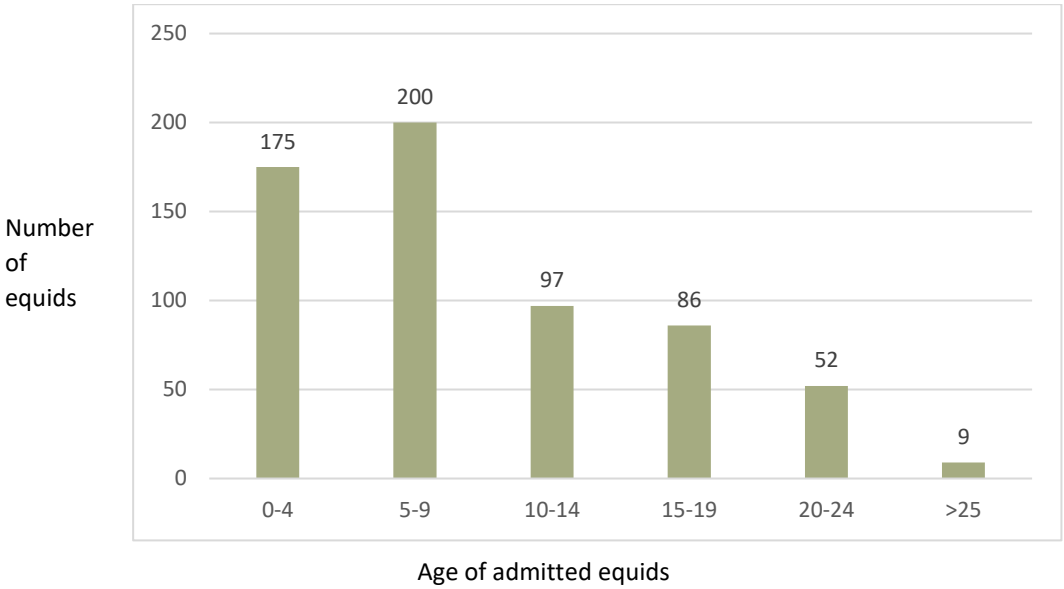


Figure 2.2: The age distribution of 619 equids that were tested for strangles in a retrospective study of equids admitted to a UK rescue centre between 2017 and 2021.

The modal breed was cob type with 44.% (n=273/617) of equids being within this category (Figure 2.3). Large ponies comprised 20.4% (n=126/617) of admitted equids, 16.0% (n=99/617) were small ponies and 8.4% (n=52/617) were light horses. The remaining breeds were donkey type, sports horse type, Arab type, or other; these made up for between 1.6% (n=10/617) and 3.6% (n=22/617) of admitted equids respectively.

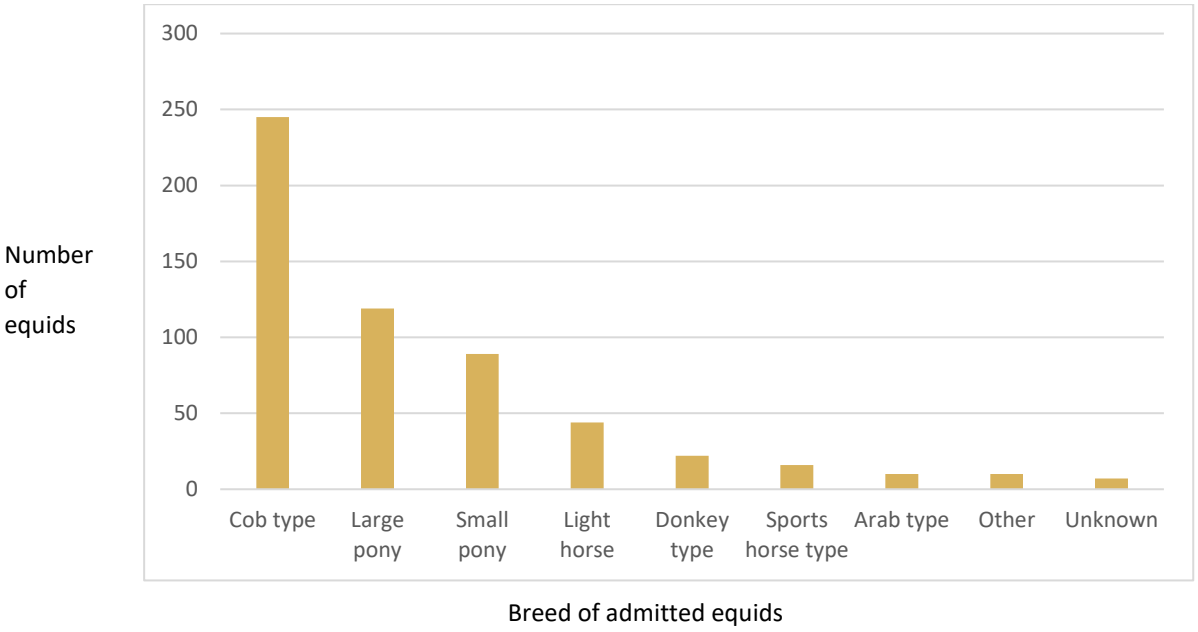


Figure 2.3: The breed distribution of 626 equids that were tested for strangles in a retrospective study of equids admitted to a UK rescue centre between 2017 and 2021.

There were 624 equids that were tested for strangles and had their sex recorded, 47.0% (n=293/624) of these equids were female and 53.0% (n=331/624) were male. Of the admitted male equids, 70.7% (n=234/331) were neutered and 29.3% (n=97/331) were entire at time of admission.

The modal body condition score was 3.0 with 25.2% (n=144/571) of admitted equids being in this category (Figure 2.4). Of these 571 admitted equids, 31.5 % (n=180/571) had a body condition score of 4.0 or over (overweight) and 29.9% (n=171/571) had a body condition score of 2.5 or below (underweight).

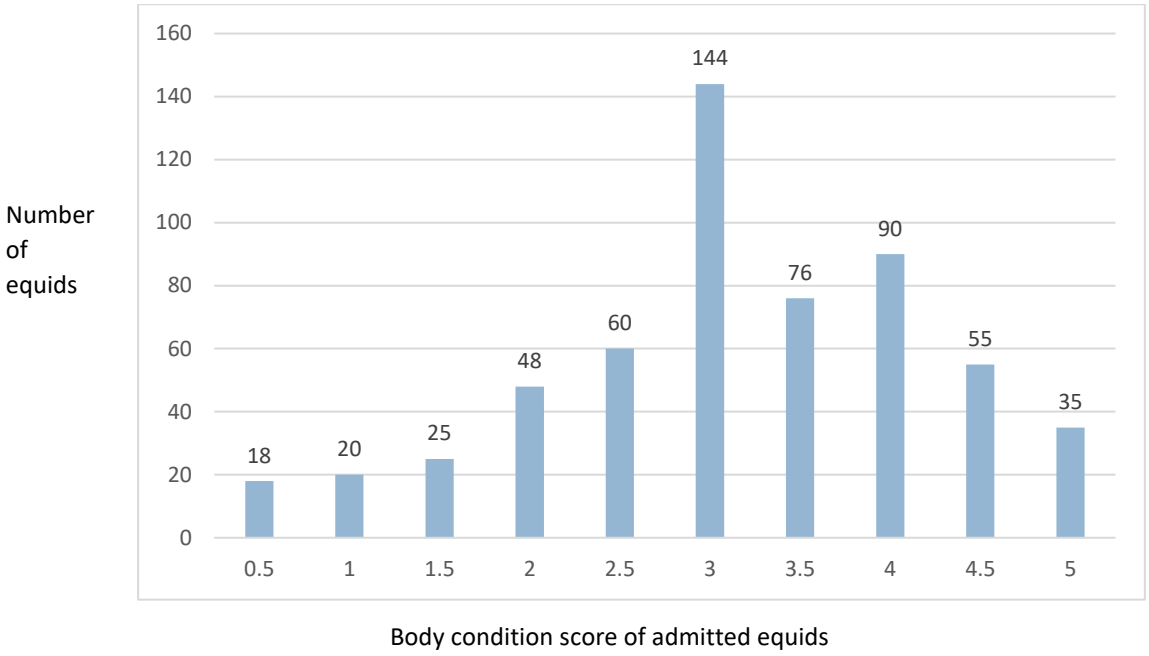


Figure 2.4: The distribution of body condition scores of 571 equids that were tested for strangles in a retrospective study of equids admitted to a UK rescue centre between 2017 and 2021.

2.3.3 Distribution of strangles cases identified between 2017 and 2021

Between 2017 and 2021, 6.1% (n=38/626) of equids with strangles were identified through the strangles screening process applied to equids on admission to Bransby Horses UK. Of the equids that tested positive for strangles, 65.8% (n=25/38) tested positive for *S. equi*, 28.9% (n=11/38) tested positive for *S. zooepidemicus*, and 5.3% (n=2/38) of equids tested negative for both *S. equi* and *S. zooepidemicus* but had chondroids within their guttural pouches (Figure 2.5).

The majority (81.6%, n=31/38) of equids with strangles were admitted during 2018 and 2019; notably fewer were admitted during 2017 (7.90%, n=3/38) and 2021 (10.5%, n=4/38) and none were admitted in 2020. The greatest number of equids with strangles were admitted during the first quarter of 2019, with 23.7% (n=9/38) of all strangles cases being admitted during this time. The largest proportion (50.0%, n=19/38) of equids with strangles were admitted during the first quarter of the years analysed; there were fewer admitted in the second (21.1%, n=8/38) and fourth (23.7%, n=9/38) quarters and only 5.3% (n=2/38)

were admitted in the third quarter. Other than in 2020, when no equids with strangles were admitted, there were always equids admitted in the first and fourth quarters.

Across all five years, 6.1% (n=38/626) of admitted equids had strangles. This proportion was highest at 11.3% (n=15/133) and 11.1% (n=16/144) during 2018 and 2019, respectively, and notably lower at 2.4% (n=3/126) and 2.8% (n=4/141) during 2017 and 2021, respectively.

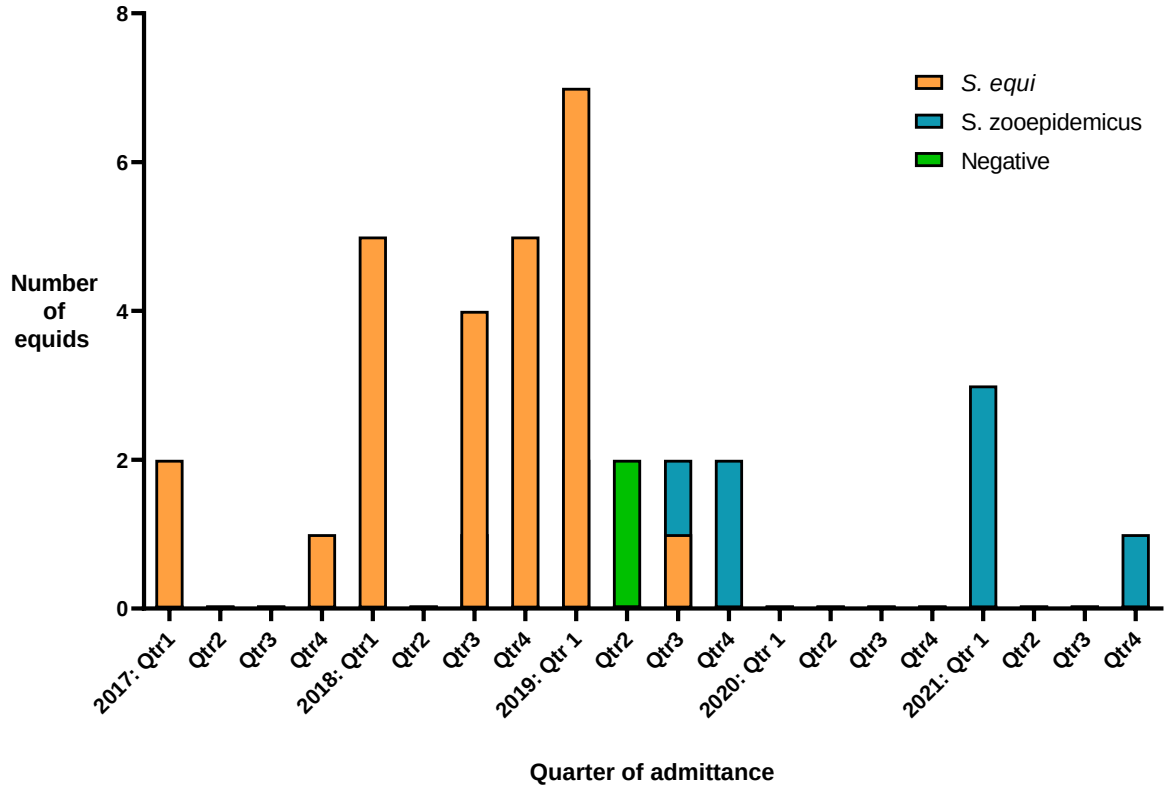


Figure 2.5: Distribution of equids with strangles that were identified through the strangles screening process of a UK rescue centre between 2017 and 2021.

The majority of equids with strangles, 63.2% (n=24/38), displayed no clinical signs associated with strangles. Chondroids were the most common clinical finding with 23.7% (n=9/38) of equids with strangles possessing them (Figure 2.6). In addition, there were two equids that had chondroids in their guttural pouches but tested negative for both *S. equi*

and *S. zooepidemicus*. Nasal discharge was present in 13.2% (n=5/38) of equids with strangles. Lymphoid hyperplasia, guttural pouch abnormalities and generalised respiratory signs were all present in 5.3% (n=2/38) of equids with strangles. Equids that tested positive for *S. zooepidemicus* had the greatest propensity to display clinical signs with 63.6% (n=7/11) of equids possessing at least one clinical sign, including 54.5% (n=6/11) having chondroids in at least one guttural pouch.

There was a statistically significant association (p=0.0021) between equids having guttural pouch abnormalities and strangles when a two-sided Fisher's exact test was performed. Guttural pouch abnormalities were observed in 13.2% (n=5/38) of equids with strangles, compared to 2.0% (n=11/561) of equids that did not have strangles. There was also an association between the presence of lymphoid hyperplasia and strangles, although this was not statistically significant when a two-sided Fisher's exact test was performed at a 95% confidence interval (p=0.0861). Lymphoid hyperplasia was observed in 5.3% (n=5/38) of equids with strangles and in 1.1% (n=6/561) of equids that did not have strangles.

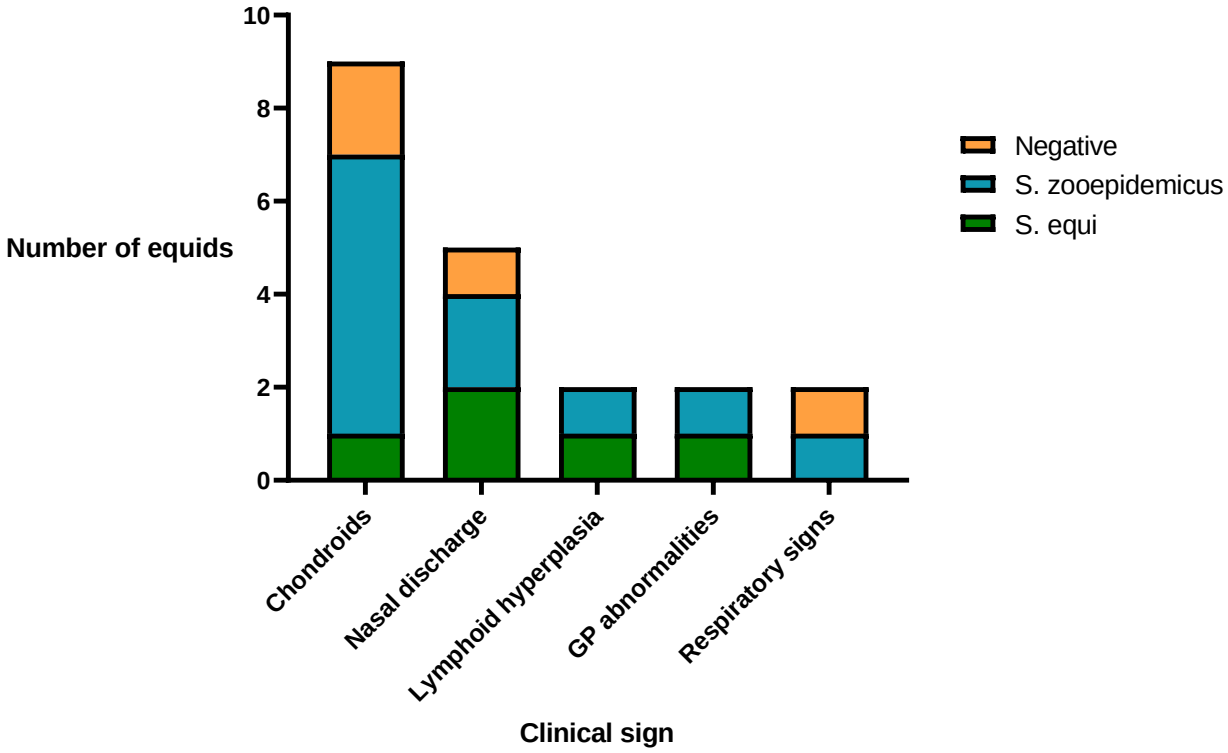


Figure 2.6: Distribution of clinical signs in equids admitted to a UK rescue centre that tested positive for *S. equi* or *S. zooepidemicus* and those with a negative test result that had chondroids in their guttural pouches.

2.3.4 Association between risk factors and strangles carriage

Four risk factors were investigated for association with strangles: age, sex, breed, and body condition (Table 2.3). Of these, age, sex, and breed were subject to statistical analysis and were not found to be associated with strangles carriage at a 95% confidence level ($p > 0.05$) when a two-sided Chi-squared test was performed.

There were not sufficient data points in each category for statistical analysis to be conducted to investigate the association between strangles carriage and body condition; although strangles carriage was more prevalent in equids with lower body condition scores. Equids with a body condition score of 0.5−2.0 had a higher proportion of strangles (15.2%, n=12/79) when compared to equids with a body condition score of 3.5−5.0 (3.80%, n=9/237).

Table 2.3: Host risk factors and their association with strangles carriage in a population of rescue equids admitted between 2017 and 2021.

Risk factor	Number of equids with strangles	Number of equids without strangles	Proportion of equids with strangles (%)	P value
Age				
0–4	12	134	8.22	**0.619**
5–9	8	180	4.26	
10–14	7	86	7.53	
15–19	6	73	7.59	
20–24	4	39	9.30	
>25	0	9	0.00	
Sex				
F	21	240	8.05	**0.133**
ME	7	70	9.09	
MN	9	214	4.04	
Breed				
Cob type	11	234	4.49	**0.353**
Large pony	9	110	7.56	
Small pony	8	81	8.99	
Light horse	3	41	6.82	
Donkey type	3	19	13.64	
Other	0	17	0.00	
Sports horse type	2	14	12.50	
Arab type	1	9	10.00	
BCS				
0.5–1.0	6	28	17.65	**NA**
1.5–2.0	6	51	10.53	
2.5–3.0	13	170	7.10	
3.5–4.0	6	152	3.80	
4.5–5.0	3	85	3.41	

BCS, body condition score; F, female; ME male entire; MN male neutered; NA, not applicable.

2.3.5 Association between biochemistry and haematology results and strangles carriage

The statistical association between biochemistry results and strangles was investigated for ten blood parameters: total protein, albumin, globulin, fibrinogen, serum amyloid A, aspartate transaminase, gamma-glutamyltransferase, creatine kinase, glutamate dehydrogenase, and alkaline phosphates (Table 2.4). The statistical association between haematology results and strangles was also investigated. This was investigated for eight blood parameters: red blood cells, packed cell volume, haemoglobin, red blood cell distribution width, platelets, white blood cells, neutrophils, and monocytes (Table 2.4). None of the tested biochemistry or haematology parameters were statistically associated ($p > 0.05$) with strangles when a two-sided Fisher's exact test was performed (Tables 2.4 and 2.5).

Table 2.4: Biochemistry results and their association with strangles carriage in a population of rescue equids admitted between 2017 and 2021.

Variable	Number of equids with strangles	Number of equids without strangles	Proportion of equids with strangles with an abnormal biochemistry parameter (%)	Proportion of equids without strangles with an abnormal biochemistry parameter (%)	P value
Total protein					
Elevated	6	76	30.0	22.4	0.417
Not elevated	14	264			
Albumin					
Lowered	4	69	20.0	20.3	>0.999
Not lowered	16	276			
Globulin					
Elevated	6	107	30.0	31.5	>0.999
Not elevated	14	233			
Fibrinogen					
Elevated	9	163	45.0	47.9	0.822
Not elevated	11	177			

Serum amyloid A					
Elevated	4	47	20.0	13.8	0.504
Not elevated	16	293			
Aspartate transaminase					
Elevated	13	175	65.0	51.5	0.259
Not elevated	7	165			
Gamma-glutamyltransferase					
Elevated	9	130	45.0	38.2	0.638
Not elevated	11	210			
Creatine kinase					
Elevated	5	65	25.0	19.1	0.560
Not elevated	15	275			
Glutamate dehydrogenase					
Elevated	4	105	20.0	30.9	0.453
Not elevated	16	235			
Alkaline phosphatase					
Elevated	2	44	10.0	12.9	>0.999
Not elevated	18	296			

Table 2.5: Haematology results and their association with strangles carriage in a population of rescue equids admitted between 2017 and 2021.

Variable	Number of equids with strangles	Number of equids without strangles	Proportion of equids with strangles with an abnormal haematology parameter (%)	Proportion of equids without strangles with an abnormal haematology parameter (%)	P value

Red blood cells					
Lowered	1	25	5.0	7.4	>0.999
Not lowered	19	315			
Packed cell volume					
Lowered	3	67	15.0	19.7	0.776
Not lowered	17	273			
Haemoglobin					
Lowered	3	85	15.0	25.0	0.426
Not lowered	17	255			
Red blood cell distribution width					
Lowered	13	207	65.0	60.9	0.816
Not lowered	7	133			
Platelets					
Lowered	2	13	10.0	3.8	0.200
Not lowered	18	327			
White blood cells					
Elevated	6	109	30.0	32.1	>0.999
Not elevated	14	231			
Neutrophils					
Elevated	2	56	10.0	16.5	0.753
Not elevated	18	284			
Monocytes					
Elevated	4	49	20.0	14.4	0.512
Not elevated	16	291			

2.3.6 Association between serological test result and strangles carriage

The statistical association between seropositivity and strangles carriage was investigated. 'Sample 1' was taken during the initial veterinary assessment and 'Sample 2' was taken at the time of guttural pouch endoscopy and lavage, typically six weeks after 'Sample 1'. 'Sample 1 or 2' refers to equids that were seropositive on one or both samples.

There was no statistically significant association ($p > 0.05$) between strangles carriage and serology result when a two-sided Fisher's exact test was performed whether a cut-off of OD ≥ 0.5 or a less stringent cut-off of OD ≥ 0.3 (Table 2.6) was used.

The dual-target iELISA targets antigens A and C; although not statistically significant, antigen A was responsible for detecting more equids with strangles than antigen C, but also generated more false positive results. This was the case when the test was performed using a cut-off of OD ≥ 0.3 or OD ≥ 0.5.

Table 2.6: Serological results for *S. equi* antigens A and C from equids admitted to a UK rescue centre between 2017 and 2021, using paired serum samples and a cut-off of OD ≥ 0.3 and OD ≥ 0.5.

	Number of equids with strangles	Number of equids without strangles	Sensitivity (%)	Positive predictive value (%)	P value[1]
Cut-off of OD ≥ 0.5					
Sample 1					
< 0.5	29	485	14.7	11.4	0.176
≥ 0.5	5	39			
Sample 2					
< 0.5	29	464	3.33	3.23	>0.999
≥ 0.5	1	30			
Cut-off of OD ≥ 0.3					
Sample 1					
< 0.3	25	410	26.5	7.32	0.524
≥ 0.3	9	114			
Sample 2					
< 0.3	25	406	16.7	5.38	>0.999
≥ 0.3	5	88			

[1]Two-sided Fisher's exact test

2.3.7 Effectiveness of strangles screening protocol

The strangles screening protocol was applied to 626 admitted equids between 2017 and 2021. During this timeframe, 38 equids with strangles were identified and treated before entering the general herd. The prevalence of respiratory disease in the general herd was evaluated between 2019 and 2021 to confirm that no equids with strangles were missed during the admission process and were spreading infection to other animals (Figure 2.7). There were 37 cases of respiratory disease identified between 2019 – 2021 with diagnoses of asthma (n=17/37), generalised respiratory signs (n=5/37) and sinusitis (n=3/37) being

most commonly reported. There were also no confirmed cases of strangles between 2017 and 2019, although this could not be retrieved from clinical records due to a change in how respiratory disease was formally recorded at the rescue centre.

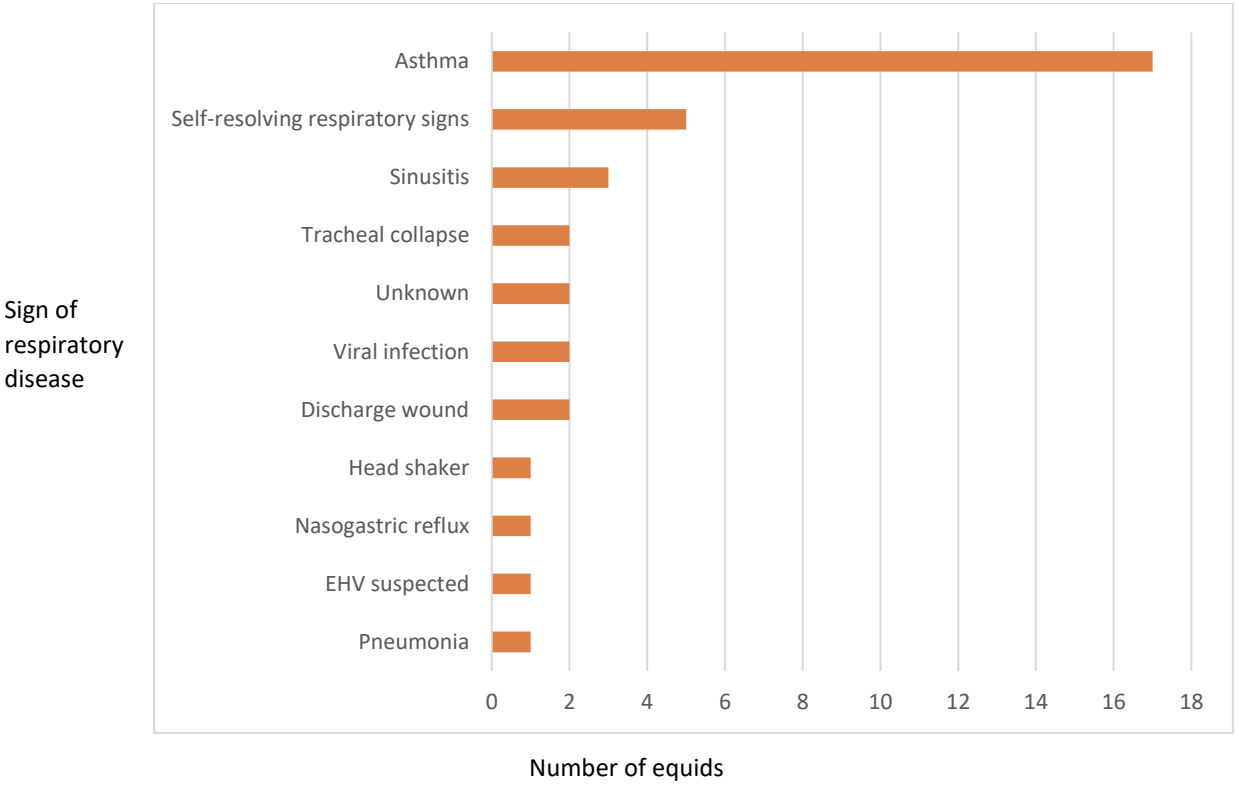


Figure 2.7: Number of equids presenting with signs of respiratory disease within a UK rescue centre between 2019 and 2021.

2.4 Discussion

2.4.1 Overview of results

This retrospective analysis aimed to determine the efficacy of a strangles screening protocol at Bransby Horses, a UK rescue centre. The studied screening protocol was implemented following a devastating outbreak at Bransby Horses UK, resulting in significant morbidity and mortality, and was caused by the presence of a strangles carrier in the herd (Harris *et al.*, 2015). By analysing clinical records and diagnostic results from horses admitted to Bransby Horses UK, insight was gained relating to factors associated with severe disease and carrier states in this unique and vulnerable population. This vulnerable population provides a unique opportunity to understand one of the most frequently diagnosed diseases of equids worldwide, with greater understanding enabling better control and prevention strategies, ultimately leading to reduced incidence and improved welfare.

The strangles-specific aspects of the screening protocol comprised paired serological tests alongside guttural pouch endoscopy and lavage. This protocol was applied to 626 admitted equids, resulting in 38 equids with strangles being identified between 2017 and 2021, a prevalence of 6.1% (n=38/626). During 2019 and 2021, there were 37 cases of respiratory disease in the main herd; as none of these cases were strangles, the screening protocol was demonstrated as working effectively at preventing carrier animals being admitted.

Chondroids, followed by nasal discharge, were the most common clinical signs in equids that were found to have strangles; although, over half the cases, 63.2% (n=24/38), were completely asymptomatic, including on guttural pouch endoscopy. There was a statistically significant association between strangles carriage and guttural pouch abnormalities and an association, although not statistically significant, between strangles carriage and lymphoid hyperplasia. Out of the equids with strangles, 28.9% (n=11/38) tested positive for *S. zooepidemicus* and these cases were more prevalent in the later years that were analysed. This finding reinforces the growing body of evidence demonstrating that *S. zooepidemicus* can act as a primary pathogen and is not truly a commensal (Waller and Wilson, 2021, Waller, 2017, Björnsdóttir *et al.*, 2017).

Guttural pouch lavage was confirmed to be effective in the detection of persistent *S. equi* infection, whereas the dual target iELISA serological test was shown to be unable to reliably

detect strangles, confirming previous reports. No host risk factors were associated with strangles carriage, reinforcing that pathogenic factors must be considered when understanding persistent *S. equi* infection. Although certain haematological parameters such as hyperfibrinogenaemia and neutrophilia have been previously linked to strangles infection (Duffee *et al.*, 2015, Hamlen *et al.*, 1994), this study found that there was no significant association between strangles carriage and any biochemistry or haematology result.

2.4.2 Implementation of screening protocol was effective

Through the enaction of the studied screening protocol, all equids with persistent *S. equi* infection were detected and treated before they entered the general herd. By preventing the infectious agent, *S. equi*, coming into contact with naïve animals, a strangles-free herd was established and maintained.

2.4.3 No association between age, sex, or breed and strangles carriage

There was no statistically significant association between strangles carriage and age, sex, or breed. It is worth noting that the study population consisted of rescue animals admitted to a welfare charity so the demographic data may not be representative of the broader UK equid population. In addition, there was limited history available for many of the equids admitted which may have introduced uncertainty in age and breed estimation, potentially leading to misclassification. It is possible that a larger data set with a greater number of strangles cases would have enabled an associated to be identified.

Age was once considered an established predisposition (Todd, 1910), but it is not consistently associated (Ling *et al.*, 2011). Todd (1910) discussed age with respect to acute *S. equi* infection, but the association is unclear beyond the fact that younger animals are more likely to be naïve to *S. equi* and may be more likely to develop the disease. Older equids might be considered to be more likely to be carriers due to having more time to develop persistent infection, but this was not found in this data set. Breed and sex are not typically considered as risk factors in the literature and were not shown to be in this study.

There was an association between body condition and strangles carriage, with equids with a lower body condition score being more like to be carriers; although, statistical analysis could not be performed due to the small sample size. Strangles has been linked to both low (Todd, 1910) and high (Ling *et al.*, 2011) body condition scores. Discussion by Todd (1910) was based on observations and not in reference to carrier animals, the existence of which were only suspected at the time. The results from Ling *et al.* (2011) were thought to be a result of equids with a higher body condition score being more likely to be in work, and mixing with other equids and thus more likely to be exposed to *S. equi*. As the study was focused on association with seropositivity, it was also suggested that equids with a lower body condition may have been less able to mount an immune response detectable by the iELISA. In this study, it may have been that equids with a lower body condition score were less able to mount an effective immune response and clear the initial *S. equi* infection, leading to persistence. Alternatively, body condition may have served as a proxy for other management-related factors that were indirectly related to *S. equi* infection and persistence. These factors could include the general welfare, husbandry, biosecurity, and levels of veterinary care received by equids, prior to their admission to this rescue centre.

Further work is required to better understand the role of immunity in adult equids, especially in carrier animals, and how different factors such as age, body condition score and exposure interact.

Overall, the lack of significant association between age, sex, or breed and strangles carriage in this study highlights the importance of developing effective screening protocols for all equids, regardless of host factors, in order to control the spread of *S. equi* and prevent outbreaks of strangles.

2.4.4 No association between haematological parameters and strangles carriage

In this study, there was no association between the biochemistry or haematology parameters investigated, 18 in total, and strangles carriage. Although not statistically significant, equids with strangles were more likely to have increases in five biochemistry parameters: total protein, serum amyloid A, aspartate transaminase, gamma-glutamyltransferase, creatine kinase. Equids with strangles were also more likely to have

lowered platelets and a lowered red blood cell distribution width and an increased monocyte count but these changes were not statistically significant. There was limited clinical history available for many equids and the prevalence of comorbidities and cofounding factors is largely unknown; it is possible that a larger, healthy, and homogenous population would have enabled an association to be identified.

Hyperfibrinogenaemia and anaemia have been linked to strangles previously (Duffee *et al.*, 2015), as have significant increases in white blood cell counts and plasma protein concentrations (Hamlen *et al.*, 1992). In the revised consensus guidelines, Boyle *et al.* (2018) stated that hyperfibrinogenaemia and neutrophilia were suggestive of strangles. Hamlen *et al.* (1992) analysed a group of 23 healthy foals that were experimentally infected with *S. equi* and Duffee *et al.* (2015) examined the clinical records of equids with confirmed strangles, including acute and persistent cases, alongside control animals. This study focused on haematological parameters in carriers, finding that these animals did not differ significantly on markers of inflammation or infection, supporting work by Pringle *et al.* (2020b). The results of this study reinforce that carrier status has minimal impact on systemic inflammation and show that haematological parameters are not a useful or reliable marker of strangles carriage.

2.4.5 Guttural pouch lavage and endoscopy recommended for carrier detection

The screening protocol consisted of a full clinical examination, comprehensive bloodwork, paired serological testing and guttural pouch lavage. Guttural pouch lavage and endoscopy was the most effective method of detecting carriers and is strongly recommended for this purpose. Guttural pouch lavage is critical to detect equids without any clinical signs of strangles carriage, evidenced by the finding that 63.2% (n=24/38) of equids with strangles in this study displayed no clinical signs associated with strangles and were only identifiable by guttural pouch lavage and microbial analysis.

Guttural pouch endoscopy is also valuable as it allows for a visual examination of the guttural pouch; this study found that two equids had chondroids in their guttural pouches but tested negative for both *S. equi* and *S. zooepidemicus*. It may be that these equids posed no risk to naïve animals; however, it is most appropriate to treat these cases as positive for

strangles and a potential reservoir of infective bacteria. Whether equids that present in this way have the potential to cause clinical disease is an avenue for future research and caution should be exercised until then. In addition, there was a statistically significant association between the presence of guttural pouch abnormalities and strangles carriage, and a non-statistically significant association between the presence of lymphoid hyperplasia and strangles carriage. These results reinforce the importance of the visual guttural pouch examination, and that blinded guttural pouch lavage may be insufficient.

2.4.6 Serological testing unable to detect carriers

The dual target iELISA serological test was shown to be unable to identify strangles carriers; in this study, there was no association between serology result and strangles carriage. Using a cut-off of OD ≥ 0.5, the sensitivity was 14.7% on the first sample and 3.3% on the second sample. Although, the specificity was much higher, 92.6% on the first sample and 93.9% on the second, this was due to the low prevalence of strangles as the vast majority of equids with strangles had a negative serological result. The difference in results between the first and second samples, taken six weeks apart, is likely reflective of equids that were recently exposed to *S. equi*; hence, the antibody levels may have fallen below the limit of detection of the iELISA. However, there was no association between serology result and strangles carriage on either sample and there were many false positive and false negative results on both the first and second tests. On the basis of this dataset, clinicians and caregivers cannot have confidence in either a positive or negative result when determining carrier status.

There are a number of reasons that may explain the failure of this test; as mentioned, this is a unique and vulnerable population, and the results may not be fully generalisable to the wider UK equid population. It may be that some carriers have such a low bacterial load, or a dormant type of infection, that insufficient bacteria are shed to stimulate an immune response detectable by this assay. Cycle threshold values were not available due to the retrospective nature of the study, little can be inferred about the bacterial load in the equids with strangles.

Mitchell *et al.* (2021) showed the international transmission of *S. equi* and described which strains are predominant around the world; it was shown that a shift from BAPS-5 to BAPS-2

has been occurring in much of Europe, including the UK, from around 2010. The dual target iELISA targets antigens A and C, both present and unchanged between BAPS-2 and BAPS-5; however, antigen C is an SeM target and *S. equi* in BAPS-2 does not produce SeM as consistently as *S. equi* in BAPS-5 does. Additionally, the genomic region encoding SeM has been shown to be subject to genomic decay in some persistent isolates (Harris *et al.*, 2015). It is notable that this study found that antigen A was responsible for detecting more equids with strangles when the test was performed using a cut-off of OD ≥ 0.3 or OD ≥ 0.5; although, it was also responsible for a greater number of false positive results. Antigen A is a segment of the gene SEQ_2190 which encodes a sortase-processed surface protein (Holden *et al.*, 2009), this gene may be lost in some persistent isolates due to genomic decay or via a SEQ_2180/SEQ_2190 recombination event (Harris *et al.*, 2015). Due to the retrospective nature of the study, isolates were not collected for sequencing to investigate whether the gene targets of these *S. equi* isolates contributed to the failure of this test.

The limitations of this serological test for carrier detection have been demonstrated previously (Durham and Kemp-Symonds, 2021, Pringle *et al.*, 2020b). However, Durham and Kemp-Symonds (2021) analysed equids at the same rescue centre as this study; as such, no new information on the generalisability of the results beyond this population can be gained. Pringle *et al.* (2020b) found that many carriers were seropositive, but equids were not managed in a way to adequately prevent re-exposure. As was found in this study, there was no significant association between seropositivity and carrier status.

Although not recommended by this study for the detection of carrier animals, serological testing has a role in the management of strangles. Single-target SeM-based iELISAs can aid in the diagnosis of purpura haemorrhagica and metastatic abscessation and can be used to screen for exposure (Boyle *et al.*, 2018, Boyle *et al.*, 2009). The dual-target iELISA can also be used to identify recent exposure and it is recommended to do so following an outbreak to identify equids exposed to *S. equi* (Boyle *et al.*, 2018, Duran and Goehring, 2021). The use of serology in screening horses being imported to the UAE was reported to prevent new incursions of *S. equi* from abroad (Craig, 2021); although the data in this current study would not support that conclusion. The difference may be due to the methodologies employed, in particular how cases were followed up, or be a result of the strains that are predominant.

2.4.7 The role of *S. zooepidemicus* in persistent infection:

A number of equids that were classified and treated as strangles carriers tested negative for *S. equi* and positive for *S. zooepidemicus*. Additionally, two equids tested negative for both *S. equi* and *S. zooepidemicus* but had chondroids in their guttural pouches. Equids that tested positive for *S. zooepidemicus* had a greater propensity to display clinical signs (e.g., chondroids), with 63.6% (n=7/11) possessing at least one clinical sign and 54.5% (n=6/11) having chondroids in at least one guttural pouch. In contrast, 20% (n=5/25) of equids that tested positive for *S. equi* displayed clinical signs and 4% (n=1/25) had chondroids. Additionally, *S. zooepidemicus* cases were detected more frequently in the later years studied; indeed, only *S. zooepidemicus* cases were detected in 2021, whereas none were detected in 2019. At present, it is unknown whether this is reflective of a wider pattern across the UK or a result of the unique intake population at this facility. All *S. equi* cases were detected by PCR, and were negative when cultured, confirming that bacterial culture is insufficient to detect *S. equi* (Lindahl *et al.*, 2013a, Boyle *et al.*, 2012, Pusterla *et al.*, 2021). In contrast, all *S. zooepidemicus* cases were detected by bacterial culture. *Streptococcus zooepidemicus* is a very diverse subspecies with the potential to cause disease across multiple species and body systems (Waller, 2017, Lindahl *et al.*, 2013b, Priestnall *et al.*, 2010, Blum *et al.*, 2010, Salasia *et al.*, 2004, Las Heras *et al.*, 2002). Further research is required to characterise the strains which cause upper respiratory disease in equids and what gene targets are optimal for identification via PCR assays.

The role of *S. zooepidemicus* in these cases is not known and questions arise over whether it is acting as a primary pathogen or secondary to *S. equi*, colonising the guttural pouch after the infection has established or after any *S. equi* present is no longer viable. Likewise, questions over the timeframe of its continued emergence are pertinent as the data in this study would suggest it is an emerging threat to equid health. *Streptococcus zooepidemicus* is known to have the potential for pathogenicity, but further research is required to determine what circumstances are necessary for it to colonise in the case of equine strangles. Even though the infective risk of a persistent *S. zooepidemicus* case is unclear, they should not be ignored and should be managed the same as a persistent *S. equi* infection.

2.4.8 Limitations of study

The primary limitation of this study was that the population analysed consisted of rescue animals admitted to a welfare charity, which may not be representative of the wider UK equid population. To address this, the population data in this study should be compared the demographic data of the population to general demographic data (BlueCross, 2018). Despite this, the population may still not be typical, which may affect the generalisability of the results. There was also a limited history available for many of the equids admitted. To reduce bias, the study had a wide inclusion criteria to retrieve and evaluate data from the clinical records of all equids admitted over the study timeframe, with only the exclusion of equids that were not screened due to transferral or euthanasia. It is also important to note that the laboratory results of the study could not be verified at a second laboratory, due to the retrospective nature of the study. Furthermore, cycle threshold values for positive PCR results were not available for many of the equids; this information would have enhanced the project by giving an indication of bacterial load, via an inverse relationship.

2.4.9 Conclusion

In conclusion, this study found no statistically significant association between age, sex, or breed and strangles carriage in the study population of rescue animals admitted to a welfare charity. There was an association between lower body condition score and strangles carriage, although statistical analysis could not be performed due to the sample size. There was no association found between biochemistry or haematology parameters and strangles carriage, and carrier status was not shown to measurably interfere with systemic inflammation. Further research is required to better understand the role of immunity in carrier animals and further characterise the current strains of *S. equi* to understand the failure of the dual-target iELISA.

The results in this study highlight the importance of creating effective screening protocols for all equids to control the spread of *S. equi* and prevent outbreaks of strangles. This specific protocol was aided by the existence of a designated quarantine unit, in which equids could be housed as they underwent the screening process, as well as an onsite veterinary team. Many equestrian facilities would be unable to replicate this, but with an understanding of the pathogenicity, transmissibility, and management of *S. equi*, it is hoped

that a similar screening protocol could be adopted by other premises. Critically, any screening protocol should include guttural pouch lavage and endoscopy, with sampled material being subject to microbial analysis (PCR and culture). Through the adoption of screening protocols, as well as other long-term control measures, such as the vaccination of unexposed animals, the control of strangles is increasingly achievable.

Although strangles carriers are typically infected with *S. equi*, some equids in this study tested positive for *S. zooepidemicus*, while others tested negative for both bacteria, but had chondroids in their guttural pouches. Equids with *S. zooepidemicus* were more likely to display clinical signs, including chondroids, and were detected more frequently in later years studied. Continuative work is needed to determine whether *S. zooepidemicus* is acting as a primary pathogen or secondary to *S. equi* and establish a management plan tailored to persistent *S. zooepidemicus* cases.

3 The *Streptococcus* of strangles: nanopore sequencing of 11 UK *S. equi* isolates

3.1 Background

The ability of *S. equi* to cause acute infection followed by long-term carriage is a major challenge for effective control and prevention of the disease. There are no host factors reliably associated with persistent *S. equi* infection; as such, there is a need to further explore the genomes of persistent *S. equi* isolates using next-generation sequencing platforms. Previous studies faced limitations associated with short read sequencing; however, long read sequencing technologies allow for the position, length, and orientation of key genomic regions to be determined.

A growing body of work has shown that the genomic elements of *S. equi* play a crucial role in its pathogenesis (Holden *et al.*, 2009, Lannergard and Guss, 2006, Tiwari *et al.*, 2007, Meehan *et al.*, 2009, Timoney and Kumar, 2008), and that genomic decay can occur in carrier isolates where genes required to cause acute disease are lost, favouring persistence within the guttural pouch (Harris *et al.*, 2015). Inversion sequences, a type of genomic rearrangement that can activate or deactivate genes based on the stage of infection, have been shown to exist in streptococcal species including *S. zooepidemicus* (Steward *et al.*, 2015), from which *S. equi* evolved (Holden *et al.*, 2009), and may play a role in the persistence of *S. equi*.

Sustained efforts to characterise the genomic elements of *S. equi* have enabled a deeper understanding of the carrier state and have formed the basis of the control of equine strangles, with key genes serving as targets for diagnostic tests and being incorporated into vaccine candidates. The genomic elements of *S. equi* are presented in the diagram below (Figure 3.1), this was generated to highlight the key virulence factors of clinical importance. The dual-target iELISA measures the immune response generated against two gene targets, antigen A (SEQ_2190, a sortase-processed surface protein) and Antigen C (N-terminus of SeM, a cell-surface M protein). Sortase-processed surface proteins are involved in the post-translational modification of proteins, including the anchoring of virulence factors to the cell wall such as SeM which is a major virulence factor in streptococcal species and is thought to contribute to immune evasion in *S. equi*. PCR assays have been designed to target *SeM*, *SeeI*,

sodA, *eqbE*, SEQ_2190 or a combination of these genes (Timoney and Artiushin, 1997, Kelly *et al.*, 2006, Baverud *et al.*, 2007, Boyle *et al.*, 2016). The commercially available triplex assay targets SEQ_2190 and *eqbE* alongside an internal control (Webb *et al.*, 2013). The Strangvac vaccine contains eight recombinant proteins, including sortase-processed proteins, cell-surface anchored proteins, and an IgG endopeptidase, fused as *CCE*, *Eq85* and *IdeE* (Robinson *et al.*, 2020). As these genomic elements are relied on for the detection and management of strangles, it is important that structural variants that could undermine effective disease control are understood and monitored.

The aim of this study was to use nanopore sequencing alongside bioinformatic analysis to investigate the presence and role of structural variants in acute and persistent *S. equi* isolates.

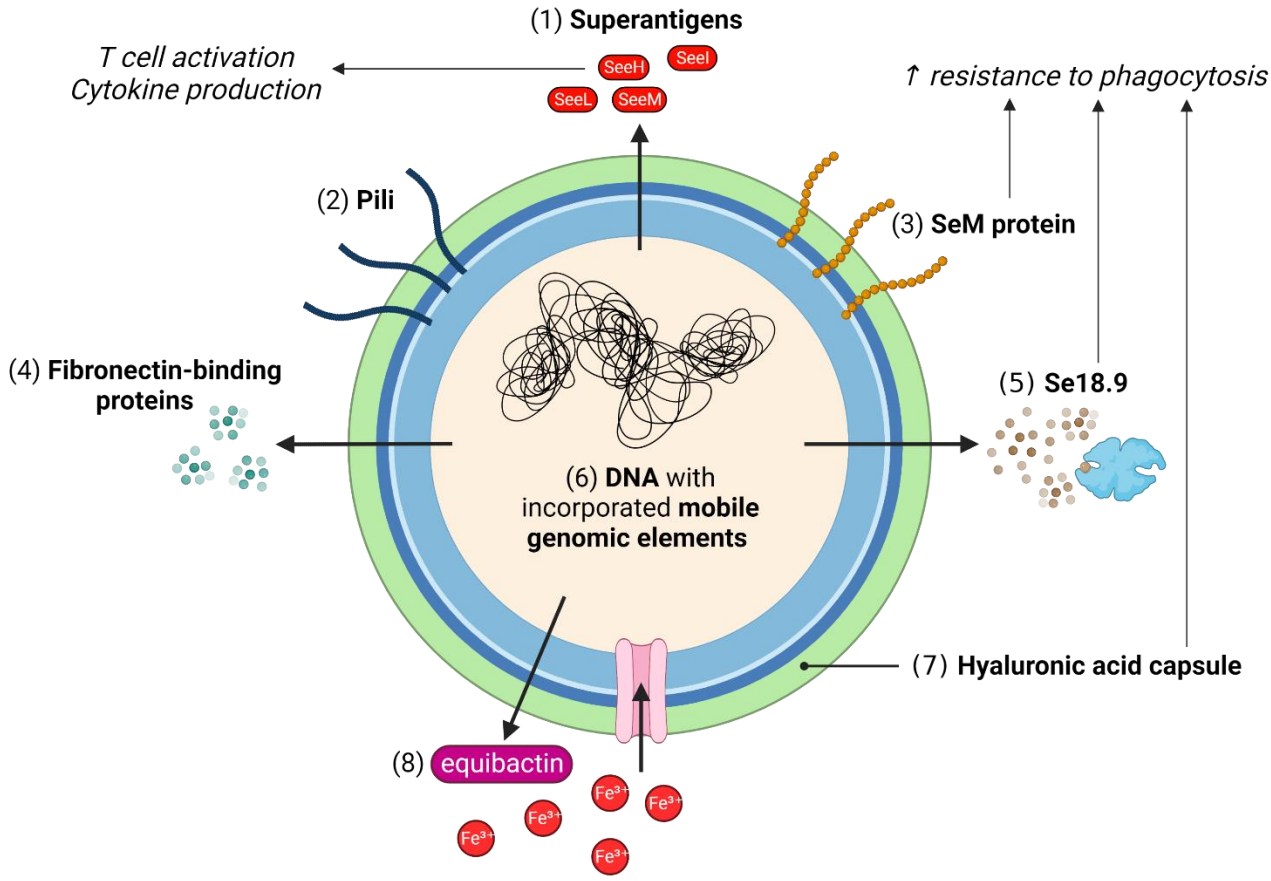


Figure 3.1 Virulence Factors of *Streptococcus equi*

(1) **Superantigens** (SeeL, SeeH, SeeI, SeeM): Inappropriately activate the equine immune system leading to T cell activation and cytokine production; (2) **Pili:** Longer pili compared to *S. zooepidemicus* to penetrate the capsule and bind to collagen more effectively; (3) **SeM protein**: Bind to fibrinogen and immunoglobulin, inhibiting C3b complement deposition. Targeted by the single and dual ELISA serological tests; (4) **Se18.9**: Secreted protein that binds to Factor H and interferes with complement activation; (5) **Fibronectin-binding proteins** (SFS, FNE): Assists with selectively binding to equine tissue and interferes with the attachment of competing pathogens such as *S. zooepidemicus*; (6) **DNA and mobile genomic elements**: The genome has acquired mobile genomic elements, such as prophage, increasing pathogenicity; (7) **Hyaluronic acid capsule**: Increased capsule depth compared to *S. zooepidemicus* enhancing resistance to phagocytosis, but reducing mucosal adherence; (8) **Equibactin:** Siderophore involved in iron acquisition, linked to the development of lymphadenopathy. Not present in *S. zooepidemicus* and lost in some carrier isolates. Created in BioRender.com by Luke McLinden

3.2 Methods

3.2.1 Isolate selection

Four acute and seven persistent *S. equi* isolates were selected for sequencing (Table 3.1). Four of the sequenced isolates were provided by A. McGlennon and the Royal Veterinary College. The other seven isolates were provided by the University of Cambridge: these

included isolate pairs from the same animal (e.g., JKS063a and JKS063c) during different stages of infection.

Table 3.1: Nanopore sequencing statistics of *S. equi* Isolates sequenced in this study to investigate genomic decay and inversion sequences.

ID	Animal sampled	Sample date	Sequencing date	Sample	Stage of disease*
JKS063a	**1**	21/09/2006	20/02/2023	NP	Acute
JKS063c	**1**	12/03/2008	20/02/2023	GPL	Persistent
JKS099a	**2**	02/11/2006	20/02/2023	NP	Acute
JKS263a	**3**	22/11/2006	20/02/2023	NP	Acute
JKS263c	**3**	02/02/2007	20/02/2023	GPL	Persistent
JKS323a	**4**	10/05/2007	20/02/2023	NP	Acute
JKS323b	**4**	05/03/2008	20/02/2023	GPL	Persistent
AX15906	**5**	23/03/2022	06/02/2023	GPL	Persistent
RA165708	**6**	01/10/2021	06/02/2023	GPL	Persistent
RO226/21a	**7**	06/04/2022	06/02/2023	GPL	Persistent
RO54407/21a	**8**	29/07/2021	06/02/2023	GPL	Persistent

GPL, guttural pouch lavage; ID, identification number; NP, nasophayngeal swab.

*stage of disease was determined by the sampling clincians on the basis of clinical information

3.2.2 Growth and extraction of bacterial DNA

For the preparation of DNA for nanopore sequencing, *S. equi* isolates were grown overnight in 10 ml of Todd Hewitt broth at 37°C in a hot air incubator.

Overnight bacterial culture (3 ml) was centrifuged (17,000 g, 5 min). The supernatant was discarded, and the harvested bacterial cells were resuspended in 500 µl of TE buffer (1M Tris-HCl, 0.5M EDTA). The samples were centrifuged (17,000 g, 5 min); the supernatant was discarded, and each cell pellet was resuspended in 400 µl of fresh cell wall disruption buffer (1M Tris-HCl, 0.5M EDTA, 30 U/ml mutanolysin and 10 mg/ml desiccated lysozyme. The samples were incubated for 30 min on a heat block at 37 °C. Bacterial cells were lysed by the addition of 22 µl SDS (20% w/v in 50mM Tris and mM EDTA) and 20 mg/ml Proteinase K and incubated overnight at 37 °C on a shaking heat block.

Saturated NaCl 6.0M (220 µl) was added to the samples and agitated for 15 s before being centrifuged (17,000 g, 10 min). Supernatant was moved to a fresh 1.5 ml Eppendorf DNA

LoBind tube. The samples were centrifuged (17,000 g, 5 min) and supernatant was moved to a fresh, 1.5 ml Eppendorf DNA LoBind tube; this step was repeated. RNAase A was added to the samples at 20 µg/µl, which were incubated for 30 min at 37°C.

DNA was precipitated by the addition of two volumes of 100% cold ethanol (stored at -20°C) and the samples were held at -20°C for 15 min. The samples were centrifuged (17,000 g, 5 min) and the 100% ethanol was replaced with 1 ml 70% cold ethanol (stored at -20°C). Samples were centrifuged (13,000 g, 5 min) and the ethanol was discarded; a further 1 ml 70% cold ethanol was added, and the samples were centrifuged (13,000 g , 5 min). Following the removal of the 70% cold ethanol, the pellet was air dried at room temperature. Once dry, the pellets were resuspended in 50 µl molecular biology grade water and allowed to resuspend overnight. To aid with resuspension, the samples were incubated for 30 min at 37°C the following morning.

3.2.3 Quantification of DNA concentration

The concentration of DNA was quantified using the Qubit dsDNA Broad Range Fluorometric Assay kit (Life Sciences, UK) following manufacturer's recommendations.

3.2.4 Preparation of DNA library and nanopore sequencing

The DNA library was prepared using the SQK-LSK109 Ligation Sequencing kit according to the protocol provided by Oxford Nanopore Technologies (Version: NBE_9065_v109+_revAD_14Aug2019), with amendments from Reiling *et al.* (2020).

Briefly, 100−200 fmol *S. equi* DNA of each sample was mixed with 3.5 µl NEBNext FFPE DNA Repair Buffer, 2 µl NEBNext FFPE DNA Repair Mix, 3.5 µl Ultra II End-prep reaction buffer and 3 µl Ultra II End-prep enzyme mix. The samples were briefly spun down in a centrifuge and incubated in a thermal cycler at 20°C for 30 min and 65°C for 5 min. An AMPure XP bead clean-up was performed according to manufacturer's instructions (Beckman Coulter), and each sample was eluted in 26 µl nuclease-free water; 80% ethanol was used to wash the sample as recommended by Reiling *et al.* (2020). At this point, 1 µl of each eluted sample was quantified using a Quibit fluorometer.

Approximately 100−200 fmol of end-prepped *S. equi* DNA was gently mixed with 2.5 µl native barcode and 25 µl Blunt/TA Ligase Master Mix (2X). This reaction was incubated for 10 min at room temperature and an AMPure XP bead clean-up was performed, and each sample was eluted in 26 µl nuclease-free water. During the end-prepping step, 80% ethanol was used as recommended by Reiling *et al.* (2020). Equimolar amounts of each sample were pooled into a 1.5 ml Eppendorf DNA LoBind tube and 1 µl of each eluted sample was quantified using a Quibit fluorometer. Since the final volume exceeded 65 µl, an additional AMPure XP bead clean-up was performed, and the pooled barcoded DNA was eluted in 65 µl nuclease-free water.

All 65 µl of the pooled barcode *S. equi* DNA was mixed with 5 µl Adapter Mix II, 20ul NebNext Quick Ligation Reaction Buffer (5X) and 10 µl Quick T4 DNA Ligase. This reaction was incubated for 10 min at room temperature and an AMPure XP bead clean-up was performed. The Short Fragment Buffer was used to wash the pellet, ensuring fragments of all sizes remained and the pellet was eluted in 15 µl elution buffer. At this point, 1 µl of each eluted sample was quantified using a Qubit fluorometer.

The library was prepared by mixing 37.5 µl Sequencing Buffer, 25.5 µl mixed Loading Beads, and 12 µl DNA library into a 1.5 ml Eppendorf DNA LoBind tube and 200 µl of priming mix was added via the priming port. The sequencing run was set to 30 h.

3.2.5 Assembly, annotation, and analysis of genome

Short read data for the corresponding isolates were parsed for quality using fastp v0.9.0 (Chen *et al.*, 2018) and default settings before being used as input for assembly using Shovill v0.9.0 (Seemann, 2007). Raw fast5 files from the long read data were base called and demultiplexed with Guppy v6.4.6 (Oxford Nanopore Technologies) using the super high accuracy configuration. The raw long read fastq files were initially aligned to the corresponding short read assembly using minimap2 v2.24 (Li, 2018) and only those reads which mapped were kept as a sam alignment file. The sam file was converted to a bam, sorted and converted back into a fastq file using samtools v1.10.2 (Li *et al.*, 2009). The high quality short and long read fastq files were used as input for hybrid assembly with Unicycler v0.5.0 (Wick *et al.*, 2017). The resulting high quality hybrid assemblies were quality checked

using BLASTn v2.9.0 (Camacho *et al.*, 2008) to infer a per contig taxonomy with the non-redundant database (Sayers *et al.*, 2022). Assemblies were visually checked using bandage v0.9.0 (Wick *et al.*, 2015). Contigs were orientated to the *S. equi* 4047 reference genome (accession number FM204883) (Holden *et al.*, 2009) using RagTag v2.1.0 (Alonge *et al.*, 2022) using the Scaffold parameters.

Annotation was achieved using Dfast v1.2.18 (Tanizawa *et al.*, 2018) with the *S. equi* 4047 reference genome (accession number FM204883) (Holden *et al.*, 2009) as a guide. Artemis v18.2.0 was used to determine the orientation of genes as well as their precise start and end positions. This information was used to generate gene arrow maps using the packages 'gggenes' v0.4.1 (Wilkins and Kurtz, 2020) and 'ggplot2' (Wickham, 2016) v3.4.1 in RStudio v4.1.1. Sourmash v4.8.0 (Brown and Irber Junior, 2019) was used to performed k-mer analysis to compare genetic similarity between genomes and generate genetic heatmaps.

3.3 Results

3.3.1 Genome statistics

Four acute and seven persistent isolates were sequenced in this study and were compared to the genome of *Se*4047 as a reference. The genomes varied in size from 2,213,351 bp to 2,288,784 bp (Table 3.2), whereas the genome of *Se*4047 was 2,253,793 bp. The number of coding sequences (CDs) varied from 2,133 to 2,279, compared to 2,137 in *Se*4047.

The complex relationships between contiguous sequences of each isolate are presented in Figure 3.2, providing a visual overview of the assembled genome.

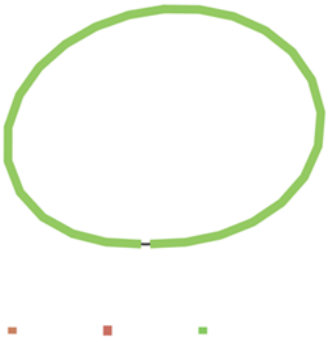
Table 3.2: Sequencing statistics of the assembled genomes of 11 UK *S. equi* Isolates sequenced using Oxford Nanopore Technologies.

ID	Contigs	Stage of disease	BP	CDs	GC content (%)	Coding ratio (%)	rRNAs	tRNAs
JKS063a	24	Acute	2247571	2191	41.1	85.2	6	28
JKS063c	28	Persistent	2215481	2133	41.2	85.1	6	31
JKS099a	17	Acute	2257279	2189	41.2	85	12	43
JKS263a	12	Acute	2257873	2205	41.1	85.4	9	42
JKS263c	14	Persistent	2268231	2199	41.2	84.9	15	52
JKS323a	18	Acute	2254491	2188	41.1	85.1	9	37
JKS323b	21	Persistent	2255124	2174	41.2	84.5	15	55
AX15906	9	Persistent	2213351	2158	41.4	84.1	15	55
RA165708	4	Persistent	2288784	2279	41.2	84.6	18	65
RO226/21a	43	Persistent	2247030	2219	41.4	83.8	12	49
RO54407/21a	3	Persistent	2267845	2271	41.2	84.5	18	65

BP, base pairs; CDs, coding sequences; CRISPRs, clustered regularly interspaced short palindromic repeats; GC context, guanine-cytosine content; ID, identification number; rRNA, ribosomal RNA; tRNA, transfer RNA.

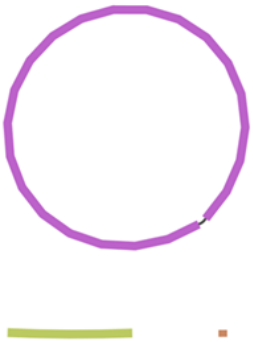
a) AX15906

b) RA165708

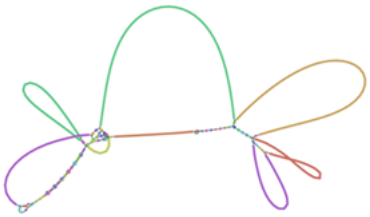


c) RO226/21a

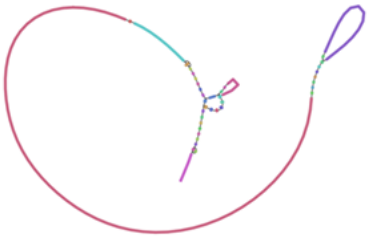
d) RO54407/21a



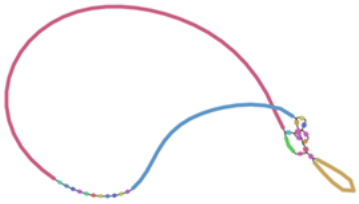
e) JKS063a



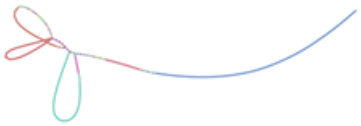
f) JKS099a



g) JKS263a



h) JKS323a



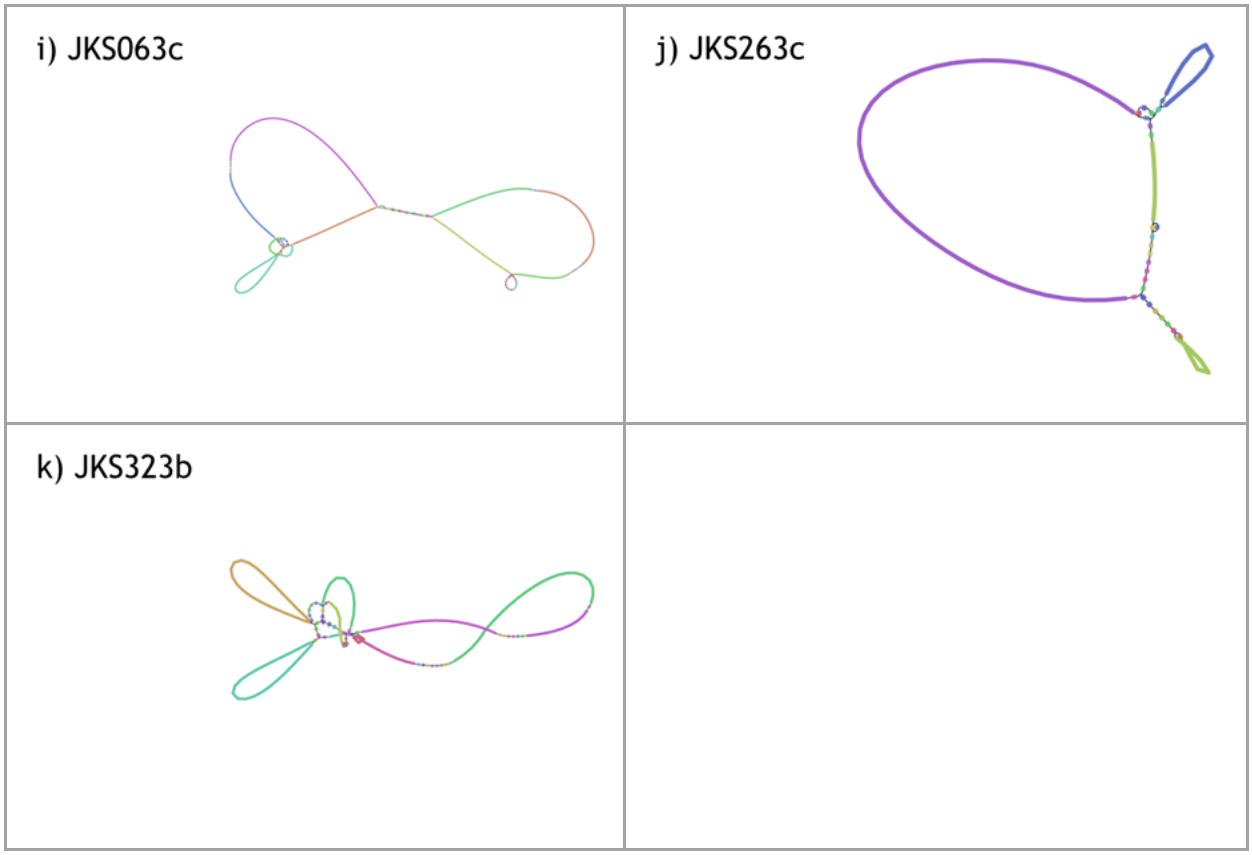


Figure 3.2: Bandage plots of 11 *S. equi* isolates showing the relationship between contiguous sequences (contigs).

A visual overview of each assembled genome is presented, showing the relationships between the contigs of each isolate. The length of each node represents the size of a contig, and the thickness of the edge represents the strength of the overlap between the nodes.

3.3.2 Genetic similarity of isolates

Seven of the sequenced isolates (JKS063a, JKS099a, JKS263a, JKS323b, JKS063c, JKS263c, JKS323b) were more genetically similar to each other, indicated by a darker colouring on the heatmap (Figure 3.3a). These seven isolates were from the same outbreak at the UK rescue centre Bransby Horses, whereas the other four isolates (AX15906, RA165708, RO226/21a, RO54407/21a) were samples from an ongoing strangles surveillance scheme (McGlennon, 2019) . The isolates RA165708 and RO54407/21a were more genetically similar to each

other than to AX15906 and RO226/21a, but these four isolates were more genetically similar to each other than to the other seven isolates (JKS063a, JKS099a, JKS263a, JKS323b, JKS063c, JKS263c, JKS323b).

The isolate JKS063c was less genetically similar to the other isolates, including the previous isolate from the same animal JKS063a (Figure 3.3b). JKS263a was also less genetically similar to JKS263c than the isolates JKS099a, JKS323a and JKS323b.

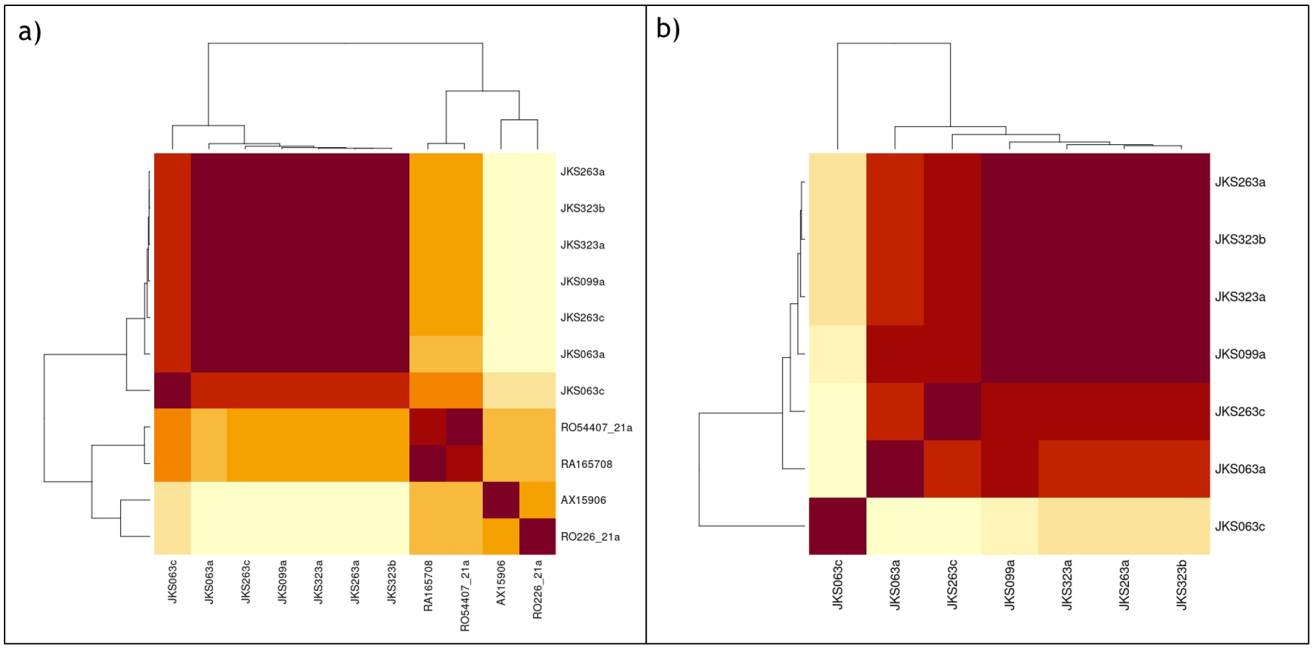


Figure 3.3: Heatmap of genetic similarity (k-mer analysis) of 11 UK isolates of *S. equi* sequenced using Oxford Nanopore Technologies, generated using sourmash.

K-mer analysis was performed on the 11 sequenced genomes. K-mers are short, fixed-length sequences of nucleotides used to compare and identify similarities between genomes. A darker colouring on the heatmap represents a greater degree of genetic similarity, whereas a lighter colouring signifies that isolates are less genetically similar.

3.3.3 Sortase-processed surface proteins

Differences in the orientation of genes were observed in one genomic region containing key sortase-processed surface proteins. In the region of genes evaluated (SEQ_2179 - SEQ_2191), containing the sortase-processed surface proteins SEQ_2180 and SEQ_2190, an

inversion in the orientation of genes was present in the isolate RO2066/21a (Figure 3.4). Furthermore, deletions were observed in three isolates; AX15906 had a deletion from SEQ_2181 – SEQ_2190, RO226/21a contained deletions in SEQ_2180 and SEQ_2190, and RO54407/21a had a deletion in SEQ_2190.

In the second region of sortase-processed surface proteins that were evaluated, SEQ_0932 – SEQ_0944 containing the key genes SEQ_0933, SEQ_0935, SEQ_0936, SEQ_0939 and SEQ_0944, inversions in the orientation of genes were not present (Figure 3.5).

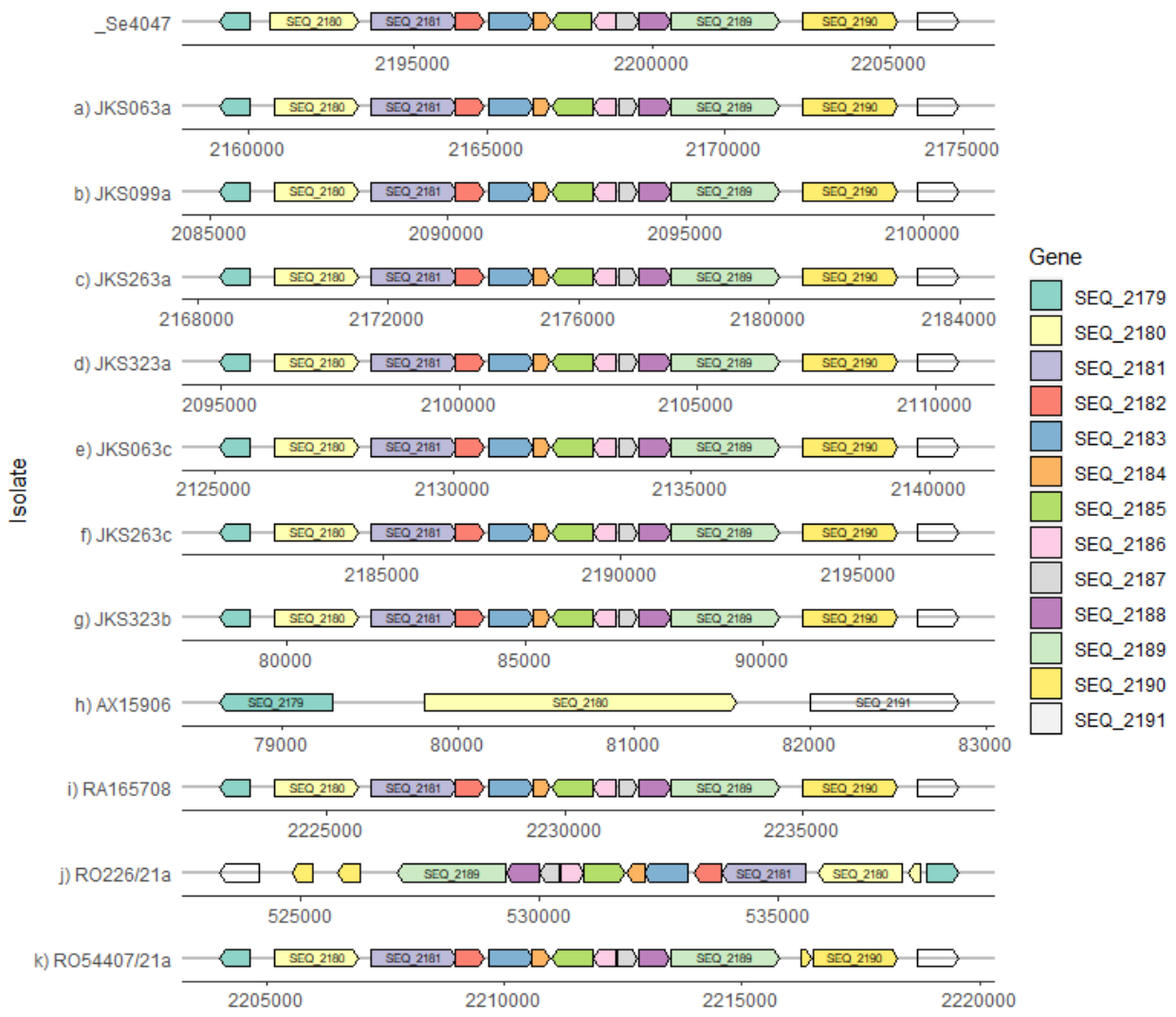


Figure 3.4: Gene arrow map of *S. equi* isolates for genes encoding and surrounding key sortase-processed surface proteins (SEQ_2179 – SEQ_2191).

Comparative gene arrow map of 11 *S. equi* isolates alongside the reference strain *Se*4047, comparing the position, length, and orientation of genes containing key sortase-processed cell surface-anchored proteins SEQ_2180 and SEQ_2190. An inversion in the orientation of genes was present in the isolate RO2066/21a. In addition, deletions were observed in three isolates; AX15906 had a deletion from SEQ_2181 – SEQ_2190, RO226/21a and deletions in SEQ_2180 and SEQ_2190, and RO54407/21a had a deletion in SEQ_2190.

Figure 3.5: Gene arrow map of *S. equi* isolates for genes encoding and surrounding key sortase-processed surface proteins (SEQ_0932 – SEQ_0944).

Comparative gene arrow map of 11 *S. equi* isolates alongside the reference strain *Se*4047, comparing the position, length, and orientation of genes containing the key sortase-processed surface proteins SEQ_0933, SEQ_0935, SEQ_0936, SEQ_0939. In all 11 *S. equi* isolates, the arrangement of genes was consistent with *Se*4047, which was used as a reference for comparison. No differences in the orientation of genes were observed in this genomic region (SEQ_0932 – SEQ_0944). In addition, no deletions or insertions were present.

3.3.4 *Streptococcus equi* M protein

In all 11 *S. equi* isolates, the arrangement of genes was consistent with *Se*4047, the reference sequence. No differences in the orientation of genes were observed in the genomic region surrounding the SeM protein (SEQ_2011 – SEQ_2022) amongst the 11 sequenced *S. equi* isolates. A 54 bp deletion in SEQ_2018 was observed in JKS263a and JKS263c. No other deletions and no insertions were observed in this region.

Figure 3.6: Gene arrow map of *S. equi* isolates for genes encoding and surrounding the SeM protein (SEQ_2011 – SEQ_2022).

Comparative gene arrow map of 11 *S. equi* isolates alongside the reference strain *Se*4047, comparing the position, length, and orientation of genes encoding and surrounding the SeM protein. In all 11 *S. equi* isolates, the arrangement of genes was consistent with Se4047, which was used as a reference for comparison. No differences in the orientation of genes were observed in the genomic region surrounding the SeM protein (SEQ_2011 – SEQ_2022) amongst the 11 sequenced *S. equi* isolates. A 54 bp deletion in SEQ_2018 was observed in JKS263a and JKS263c. No other deletions and no insertions were observed in this region.

3.3.5 Hyaluronic acid capsule

No differences in the orientation of genes were observed in the genomic region comprising the *has* locus (SEQ_2068 – SEQ_2074) amongst the 11 sequenced *S. equi* isolates (Figure 3.5). Deletions were observed in this locus; a partial deletion of *hasA* (SEQ_0269) was present in the isolate RA165708 and a partial deletion of *hasB* (SEQ_0270) was present in the isolate AX15906.

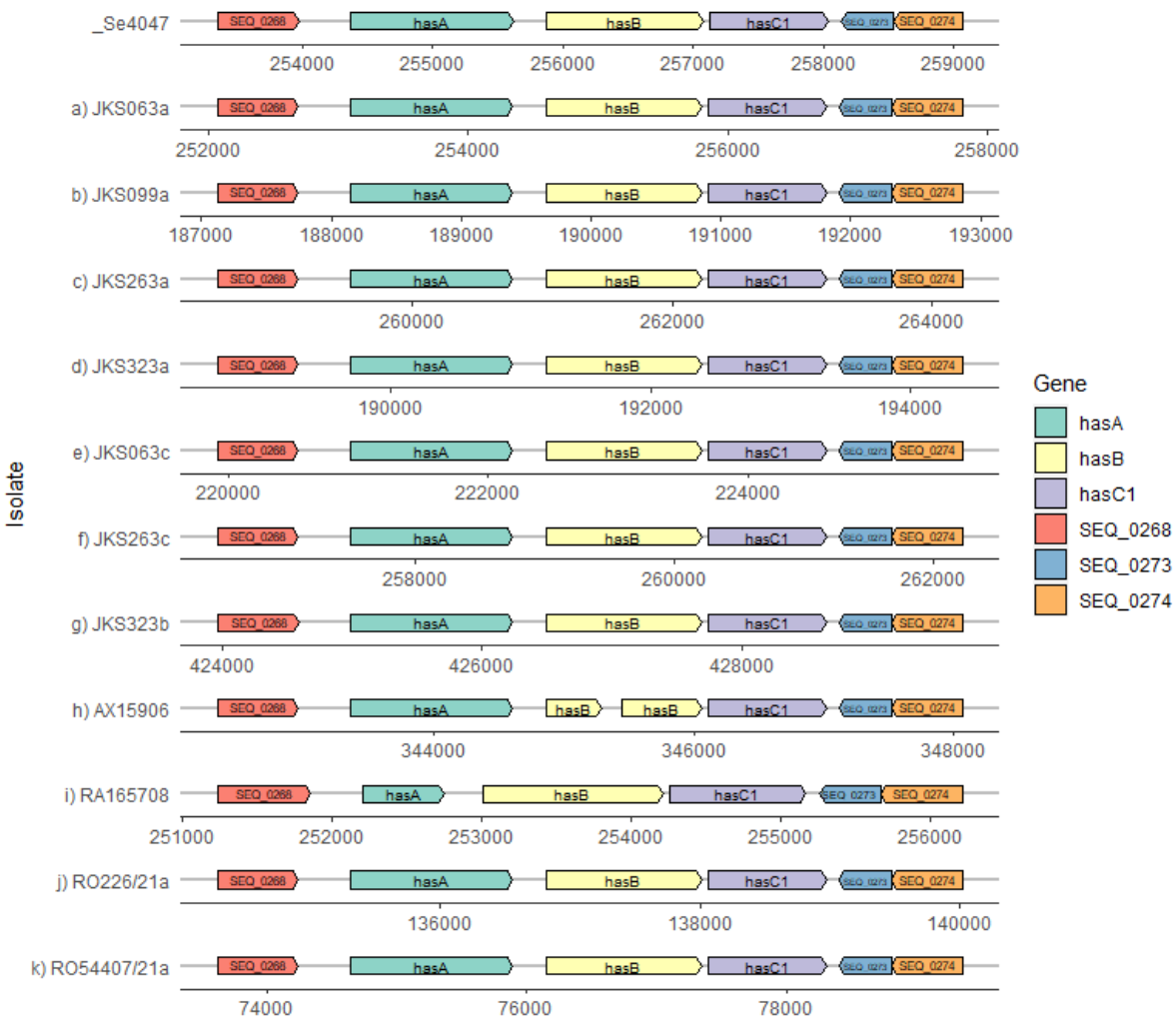


Figure 3.7: Gene arrow map of *S. equi* isolates for genes encoding and surrounding the hyaluronic acid capsule (SEQ_0268 – SEQ_0274).

Comparative gene arrow map of 11 *S. equi* isolates alongside the reference strain Se4047, comparing the position, length, and orientation of genes encoding and surrounding the hyaluronic acid capsule. No differences in the orientation of genes were observed in this genomic region (SEQ_0268 – SEQ_0274) amongst the 11 sequenced *S. equi* isolates. Deletions were observed in this locus; a partial deletion of *hasA* (SEQ_0269) was present in the isolate RA165708 and a partial deletion of *hasB* (SEQ_0270) was present in the isolate AX15906.

3.3.6 Integrated phage within *has* locus

Six isolates were found to contain a phage, integrated within the genes encoding and surrounding the hyaluronic acid capsule (Figure 3.8). This included isolates recovered from

horses in both acute (JKS063a, JKS099a, JKS263a, JKS323a) and persistent (JKS263c, JKS323b) stages of *S. equi* infection. In all six isolates, the phage was integrated between genes SEQ_0267 and SEQ_0268 and was 33,045 bp in length and encoded 53 genes.

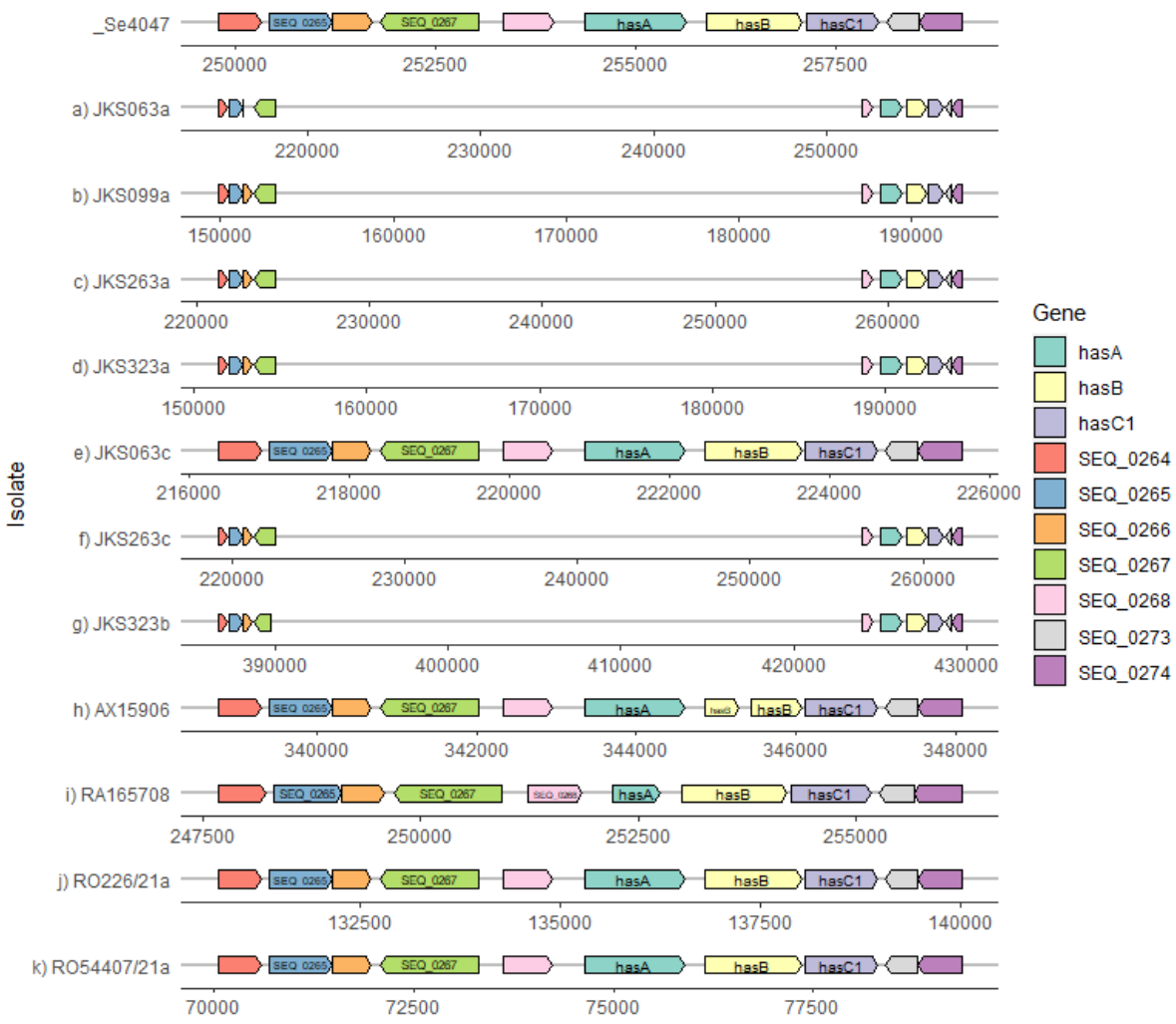


Figure 3.8: Gene arrow map of *S. equi* isolates for genes encoding and surrounding the hyaluronic acid capsule (SEQ_0264 – SEQ_0274).

A gene arrow map of 11 *S. equi* isolates alongside the reference strain *Se*4047, comparing genes encoding and surrounding the hyaluronic acid capsule (SEQ_0264 - SEQ_0274). No differences in the orientation of genes were observed in this genomic region (SEQ_0268 – SEQ_0274) amongst the 11 sequenced *S. equi* isolates. Deletions were observed in this locus; a partial deletion of *hasA* (SEQ_0269) was present in the isolate RA165708 and a partial deletion of *hasB* (SEQ_0270) was present in the isolate AX15906. Six isolates were found to contain a 30,054 bp integrated phage within this locus.

3.4 Discussion

3.4.1 Overview of results

This study used Nanopore sequencing to analyse the genome of *S. equi*, focusing on key loci that are required for virulence or targets of diagnostic tests and vaccine candidates. Structural variants, including deletions, insertions, and inversions, were observed in key genomic regions of *S. equi*. Deletions in persistent isolates were found in regions coding for sortase-processed surface proteins (SEQ_2190 and surrounding genes) and in the genomic region that encodes the hyaluronic acid capsule. These findings provide evidence of genomic decay in persistent isolates of *S. equi* confirming previous reports (Harris *et al.*, 2015, Morris *et al.*, 2021). An integrated phage was responsible for a 33,045 bp insertion within the *has* locus in six isolates; although, the effect of this insertion is unknown. In addition, an inversion in a key genomic region encoding the sortase-processed surface protein SEQ_2190 was confirmed in *S. equi* for the first time, demonstrating that persistence within the guttural pouch is a more dynamic and complex process than previously thought.

3.4.2 Genomic decay in persistent *S. equi* isolates

A number of deletions were found in this study; notably, two isolates (AX15906, RA165708) had deletions in the *has* operon. RA165708 had a deletion in *hasA* which codes for hyaluronan synthase and AX15906 had a deletion in *hasB* which codes for UDP-glucose 6-dehydrogenase; both are thought to be necessary for production of the hyaluronic acid capsule. This polysaccharide structure is a key determinant of virulence in acute isolates *S. equi*, reducing phagocytosis and making the infection harder to clear (Holden *et al.*, 2009, Woolcock, 1974, Wessels *et al.*, 1991, Timoney *et al.*, 2014). A deletion in this locus could result in reduced virulence with a loss or reduction in the beneficial effects on pathogenicity associated with the capsule, resulting in a lessened ability to infect other equids. In addition, reduced capsule depth in *S. equi* could in greater susceptibility to the host immune response, relying on the response waning sufficiently by this point. However, an attenuation of this locus could facilitate persistence by providing biochemical advantages and aiding in adaption to the guttural pouch environment, granting improved adherence to host mucosal tissues or increased resistance to environmental stresses.

Deletions in the genomic region containing the sortase-processed cell surface-anchored proteins SEQ_2180 and SEQ_2190 were present in three isolates (AX15906, RO226/21a and RO54407/21a). RO226/21a and RO54407/21a had deletions in SEQ_2190, RO226/21a had a deletion in SEQ_2180 and AX15906 had a deletion from SEQ_2181 − SEQ_2190. SEQ_2180 and SEQ_2190 are both sortase-processed cell surface-anchored proteins. The precise functions of these proteins are unknown but could be linked to adhesion, invasion, or immune evasion. Sortase-mediated anchoring is widely used by Gram-positive bacteria to attach a variety of proteins to the cell wall, including adhesins, pili, and other surface proteins involved in bacterial virulence and host colonisation. Deletions in the genes encoding and surrounding these proteins could have a profound effect on the ability of *S. equi* to colonise and evade the equine immune system. The result could be bacteria less able to infect other equids, but possessing genomes better adapted to persistence.

These findings build on work by Harris *et al.* (2015) in which deletions in the *has* locus, as well as deletions in the equibactin locus, and truncation of the N-terminal region of the SeM protein, a major virulence factor of *S. equi*, have been linked to strangles carriage (Chanter *et al.*, 2000). Morris *et al.* (2021) identified no consistent changes in the genomes of isolates from carrier horses; likewise, deletions were not consistently observed in persistent isolates in this study. However, in Morris *et al.* (2021) the genomes of some isolates recovered from persistently infected horses were found to contain deletions in *SeM* and the citrate locus. The aim of this study was not to evaluate a large number of isolates for consistent changes, but to harness long-read sequencing techniques to evaluate a smaller number of isolates; as such, it is not surprising that deletions were not consistently observed across the small number of isolates recovered from persistently infected equids.

The results of this study and others (Morris *et al.*, 2021, Harris *et al.*, 2015) provide substantial evidence of genomic decay in persistent isolates of *S. equi*, highlighting that different gene repertoires may be advantageous during long-term persistence or acute disease. Deletions have also been observed in acute isolates; these acute isolates may be less virulent than other strains of *S. equi*, but sufficiently 'fit' to cause recurrent outbreaks in unexposed animals (Tscheschlok *et al.*, 2018). The differences in the incidence and location of the specific structural variants observed are potentially reflective of the varied selective

pressures that are exerted on *S. equi* as this organism persists within the guttural pouches of different horses.

3.4.3 Inversions present in key *S. equi* genes

In this study, inversion sequences were confirmed in *S. equi* for the first time, emphasising the dynamic nature of the *S. equi* genome. Inversions were found in a locus encoding the sortase-processed cell-surface anchored proteins SEQ_2180 and SEQ_2190. As discussed, the functions of these sortase-processed cell surface-anchored proteins are unknown but likely linked to adhesion, invasion, or immune evasion. Inversions in this locus could result in the attenuation of these genes and could contribute to the reduced virulence and transmissibility often observed in persistent isolates of *S. equi*. In addition, SEQ_2190 is a target in the dual-target iELISA; inversions in isolates recovered from carrier animals could contribute to the observed failure of this diagnostic test (Durham and Kemp-Symonds, 2021).

Inversion sequences are present in a number of streptococcal species including *S. pyogenes* and *S. pneumoniae* with phenotypic effects on capsule and pilus production (Li and Zhang, 2019), and a beneficial impact on persistence and adaptation (Bao *et al.*, 2016). Phase variation has been observed in *S. zooepidemicus* and *S. suis*. In *S. suis*, methyltransferases have been shown to enable biphasic on-off switching via recombination-driven shuffling or simple sequence repeats (Atack *et al.*, 2018). In *S. zooepidemicus*, the existence of subpopulations within an environment confers survival advantages and ensures it is a versatile opportunistic pathogen; Waller (2017) describes a population in which 80% had a DNA segment in one orientation and 20% had it in the reverse orientation. The reversible region of DNA acted as a switch that controlled the production of the sortase-processed cell surface-anchored protein, SZO08560 (Steward *et al.*, 2015). A similar result may be achieved in *S. equi* by the inversion of the loci described in this study, with subpopulations existing simultaneously and in conjunction with each other within the environment of the guttural pouch, better allowing for continued survival. These findings present *S. equi* as a more dynamic and potentially versatile pathogen than previously thought, as a result of its genomic plasticity.

3.4.4 Javan phage integrated within *has* locus

An integrated phage (prophage) first identified by Rezaei Javan *et al.* (2019) was found in six isolates, integrated within genes encoding the hyaluronic acid capsule. The prophage was found to have an integrase at the 5' terminus which was likely the insertion mechanism, and a transcriptional regulator at the 3' terminus, which could have been responsible for excision. The acquisition of this prophage, an example of an MGE, reflects the plasticity of the *S. equi* genome; the gain of MGEs is thought to play a crucial role in the evolution of *S. equi* from *S. zooepidemicus*, resulting in its larger genome (Holden *et al.*, 2009).

Four isolates containing the prophage were sampled from horses with acute *S. equi* infection (JKS063a, JKS099a, JKS263a, JKS323a), while the remaining two were isolated from samples obtained from two of these horses at a later date when the infection had become persistent (JKS263c, JKS323b). JKS263a and JKS263c, as well as JKS323a and JKS323b, were samples taken from the same animals during different stages of infection. All isolates from the same outbreak at Bransby Horses UK in Lincolnshire. It is notable that the other four isolates from animals with persistent infection (AX15906, RA165708, RO0226/21a and RO54407/21a) were not found to contain the prophage; although, they were from different outbreaks across the UK and the samples were taken several years later. The isolate JKS063a was found to possess the prophage, while JKS063c, a strain retrieved from the same horse at a later time period, lacked this prophage. This finding provides evidence of prophage loss during the progression from acute to persistent infection.

Prophage are widespread in streptococci (Rezaei Javan *et al.*, 2019) and are responsible for a range of phenotypic effects, including increasing host fitness, immune evasion, and virulence (Casjens, 2003, McShan *et al.*, 2019). Their propagation is dependent on the continued persistence of the bacteria, so their acquisition can promote host fitness and virulence, but their effects can also be detrimental. The hyaluronic acid capsule has been linked to pathogenicity and immune evasion, through phagocytosis resistance enhancing survival, and the increased depth in *S. equi* compared to *S. zooepidemicus* is thought to contribute to the increased pathogenicity of *S. equi* (Holden *et al.*, 2009). It is possible that the insertion could enhance the functionality of the *has* operon or attenuate it by disrupting

the sequence; assessing hyaluronic acid capsule depth on growth media could determine the effect of this prophage on the *has* operon.

The finding that all acute isolates evaluated in this study were found with this prophage could be of significance. However, the outbreak from which all isolates containing the prophage were taken, the Lincolnshire outbreak described by Harris *et al.* (2015) and Mitchell *et al.* (2021), was likely caused by a pre-existing carrier. Therefore, the strain responsible for causing acute disease may still have attributes of a carrier strain. This outbreak is not reflective of the global *S. equi* population and more recent outbreaks (ST-151) are not closely related to this strain (ST-179) (Mitchell *et al.*, 2021); indeed, the prophage may have a detrimental effect that contributed to the decline of this strain. Regardless of the specific effects of the phage, the plasticity of the *S. equi* genome is highlighted and differences between acute and persistent isolates are evident. This flexibility may contribute to the persistence of *S. equi*, with genomic elements that aid in causing acute disease being lost as the animals become carriers or result in these less virulent strains going down an evolutionary dead-end and being replaced by fitter strains.

3.4.5 Implications and future research

The results of this study highlight the potential importance of structural variants such as inversions, insertions and deletions when considering the persistence and control of equine strangles. One genomic region that was subject to both inversions and deletions (SEQ_2179 to SEQ_2191) is a target of diagnostic tests including the dual-target iELISA triplex PCR assay (Webb *et al.*, 2013); the implications of these genomic changes must be determined to assess the impact on strangles control. Structural variants were not observed in other regions relevant to clinical practitioners, such as *eqbE* in the equibactin locus which is in the triplex PCR assay or *CNE* which is in the Strangvac recombinant fusion protein vaccine, providing reassurance that these targets can be relied upon.

These samples were not selected to be representative of the global *S. equi* population and little information on the prevalence of structural variants can be gained from this analysis. Future work, with more samples with a wider geographic origin, will allow the clinical implications of these findings to be determined, including how inversions may affect

diagnostic testing and vaccination. Analysis of the transcriptome, methylome and proteome would yield information on the effect of inversion sequences and their role in the pathogenesis and persistence of *S. equi*. Analysis of sub-populations within the guttural pouch, as well as the larger microbiome, would aid in determining the prevalence and impact of inversion sequences within a population as well as the interaction between sub-populations. The true role, prevalence, and implications of the Javan phage found here must be studied further, in particular, to assess whether this prophage is linked to increased (or decreased) virulence and pathogenicity.

3.4.6 Limitations of study

Bacterial persistence can involve subpopulations with different genotypic and phenotypic characteristics (Steward *et al.*, 2015, Waller, 2017) and these findings represent only a snapshot of the diversity and dynamics within this environment. As a result, a degree of caution is advised when extrapolating these results to the wider, global population of *S. equi*. In addition, a relatively small number of samples with the same geographic origin (UK) were sequenced, which may affect the generalisability of the results. Of the 11 isolates that were sequenced, the four collected as part of an ongoing surveillance scheme (McGlennon, 2019) represent strains from different outbreaks across the UK. The remaining seven isolates were from a single outbreak in Lincolnshire allowing acute and persistent isolates from the same animals to be examined. All 11 isolates had been previously sequenced using the Illumina platform, allowing for hybrid genome assembly; this addressed the high base-calling error associated with Nanopore sequencing.

3.4.7 Conclusion

This study has provided important and novel insights into the genomic features of *S. equi* that may contribute to persistence in the equine guttural pouch. Evidence of genomic decay is provided, with deletions being found in persistent isolates in genes encoding sortase-processed surface proteins and the hyaluronic acid capsule. Inversions sequences were identified in *S. equi* for the first time in a key locus that has been linked to virulence and pathogenicity. A Javan phage integrated within the *has* locus was found in six isolates,

including all four acute isolates sequenced, suggesting this phage could have an effect on virulence, but further research is required to confirm this.

This study demonstrates that the genome of *S. equi* is not static, but evolves in an incredibly dynamic and complex process, about which there is much still to learn. There were clear genetic differences between acute and persistent isolates and a number of deletions in persistent isolates. However, the presence of insertions and inversions imply that the process is more nuanced and that a simplistic delineation does not reflect the complex environment. It is possible that persistence within the guttural pouch may rely on the interaction between different sub-populations, contributing to the dynamic nature of persistence. Overall, this study provides a foundation for future research alongside revealing novel insights which contribute to the understanding of the persistent, and increasingly preventable, threat of equine strangles and the carrier state.

4 Discussion: understanding the persistence of strangles

4.1 Overview of results

Equine strangles has been able to circulate for hundreds of years due to the presence of carrier animals, in which *S. equi* persist, spreading the infection to naïve animals. Since the carrier state was confirmed (Newton *et al.*, 1997a, George *et al.*, 1983), it has been an area of active research; however, it is not known why only about 10% of equids become carriers following an infection. It is speculated that the process of becoming a carrier is multifactorial and dependent on the interplay between the host and bacteria as well as local conditions and related management factors. In this project, host and pathogenic factors were investigated to better understand persistent *S. equi* infection.

In this study, the carrier state was shown to be more complex and dynamic than once thought, with implications for the detection and management of equine strangles. No haematological parameters were statistically associated with strangles carriage in the retrospective clinical study, demonstrating the minimal effect strangles carriage has on systemic inflammation. In addition, there were no predictor variables for persistent infection, confirming the 'silent' nature of carriage. When acute and persistent isolates of *S. equi* were investigated in the molecular study, a number of structural variants including inversions, insertions and deletions were observed in key loci, demonstrating the intricate nature of persistence, which suggests potential for sub-populations and microenvironments within the guttural pouch. Inversions, insertions, and deletions were seen in genes that are targets of diagnostic tests or vaccines; although, the clinical implications of these structural variants must be determined.

4.2 Detection of carrier animals

4.2.1 Carriers and economics

The problems associated with the detection of carrier animals are a key reason for the continued persistence of equine strangles. This study has confirmed that guttural pouch lavage and endoscopy is the most effective method of carrier detection, providing the evidence to validate it as the 'gold-standard' method. No method of diagnosis is infallible, but a visual assessment of the guttural pouch combined with PCR and culture of material obtained by lavage is the current best method to detect equids with persistent *S. equi* infection.

Although preventing strangles outbreaks through the detection of carrier animals will always be preferable to managing an outbreak after it has erupted, there are many barriers to the implementation of effective screening protocols. Bransby Horses UK has a unique set-up and have invested significantly in strangles prevention following a devastating outbreak in 2008. This investment included the creation of a dedicated quarantine unit, in which equids can be housed until they have been screened for strangles and other infectious diseases. They also have an onsite veterinary team and with their own endoscopic equipment. A dedicated set-up of this nature can only exist in large equestrian facilities where significant financial investment is possible. Screening protocols can still exist in smaller equestrian facilities, but there are issues around cost, practicality, enforcement, and owner compliance. Equids that travel and mix with other animals frequently would have a greater risk of exposure to *S. equi* but strangles screening following every external trip is unlikely to be an option. For these animals, the Strangvac vaccine may provide sufficient protection against *S. equi* to prevent strangles infections establishing and spreading to other equids. Vaccination with Strangvac generates immune responses against eight proteins of *S. equi*, conferring up to 94% protection against experimental challenge (Robinson et al., 2020) and there is evidence of reduced shedding from ponies that were vaccinated with Strangvac (Waller, 2023). In addition, there was shown to be conservation in the antigenic sequences of the Strangvac vaccine and 254 diverse isolates of *S. zooepidemicus*, with Frosth et al. (2023) demonstrating that 89% of *S. zooepidemicus* isolates encoded at least four antigens with >70% amino acid identity. These results would suggest there is a level of cross-

protection against *S. zooepidemicus* infection provided by Strangvac, and that *S. zooepidemicus* may act as a natural booster for the vaccine in the field.

4.2.2 Diagnostic and vaccine targets

Guttural pouch lavage relies on microbial analysis, as do nasopharyngeal and nasal swabbing techniques that were not evaluated in this study. Therefore, diagnostic targets must be monitored and updated if necessary; *SeM* was once the primary target in PCR assays (Timoney and Artiushin, 1997), but other targets are now more commonly used such as *eqbE* and SEQ_2190 which, are the targets of the commercially available triplex assay, alongside an internal control (Webb *et al.*, 2013). If these targets are not optimal then the efficacy of diagnostic methods that rely on PCR analysis, and any screening protocol that employs them, could be undermined. Fortunately, structural variants of *eqbE* and other genes in the equibactin locus were not found in this study; although, these genes were subject to genomic decay in persistent isolates in previous work (Harris *et al.*, 2015). Inversions and deletions were identified in the genomic region encoding and surrounding the gene SEQ_2190 in persistent isolates in this study. The implications of these structural variants are unclear and should be an area of future research. Having multiple gene targets within an assay mitigates issues associated with each target or variation in expression between strains of *S. equi*. In the second chapter, PCR was shown to be effective at detecting *S. equi* and there is no published evidence for failure of this PCR assay. However, the importance of a functional PCR assay is such that this potential issue should continue to be monitored to assess whether they are compromised by deletions or inversions in the genome of *S. equi*.

One genomic region evaluated in this study encodes the fibrinogen-binding protein *CNE*, a protein subunit in the Strangvac vaccine (Robinson *et al.*, 2018). No structural variants were seen in the genes encoding and surrounding this sortase-processed surface protein. This finding is notable as it provides reassurance to veterinary professionals when recommending this vaccine to owners. Furthermore, the Strangvac vaccine consists of eight protein subunits (Robinson *et al.*, 2020), so changes in an individual target are unlikely to dramatically affect the generation of a sufficiently robust immune response.

4.2.3 Role of serological testing

Serological testing is often used for carrier detection due to economic and practical reasons as guttural pouch lavage is prohibitively expensive for many. There is evidence for the failure of the dual-target iELISA to detect carrier animals (Durham and Kemp-Symonds, 2021, Pringle *et al.*, 2020b) and the limitations of the assay were evident in this study. This study evaluated equids at the same UK rescue centre as Durham and Kemp-Symonds (2021), but a greater number of admitted equids over a longer period of time were analysed. The dual-target iELISA consists of two targets, portions of SEQ_2190 (Antigen A) and SeM (Antigen C). A 54 bp deletion was observed in SEQ_2018, a gene adjacent to *SeM*, and truncated *SeM* sequences have been demonstrated in carrier animals elsewhere (Chanter *et al.*, 2000). In persistent isolates, deletions and inversions were seen in SEQ_2190 and surrounding genes; these structural variants could help explain the failure of the iELISA to detect carrier animals. The clinical implications of deletions and inversions in this locus and *SeM* must be determined. If they have an effect on the expression of the proteins these regions encode, this could interfere with the host's ability to form antibodies against these proteins. The iELISA would then be unable to detect a measurable serological response directed against these immunodominant proteins. This is an example of how understanding the complexities of persistence within the guttural pouch has an impact on the detection and management of equine strangles. If these targets are not reliably expressed in persistent isolates of *S. equi*, then it is not appropriate for them to be the target of diagnostic tests that are relied upon by clinicians.

Regardless of the reason, this iELISA has been shown to be unable to reliably detect carrier animals; consequently, it cannot be recommended for this purpose. It is notable that Bransby Horses UK continue to use the dual-target iELISA to inform decision-making around husbandry and biosecurity prior to guttural pouch lavage and endoscopy. However, the data presented in the second chapter shows that there is no association between serological status and carrier status, and its use should be reserved for detecting recent exposure. The iELISA was not developed to provide a cheaper and easier alternative to diagnosing carriers, but as a tool to screen for exposure to *S. equi* and it should continue to be used in this way. This is especially true when determining which equids have been exposed following an

outbreak. The iELISA can direct clinicians to conduct further investigations in exposed animals by performing guttural pouch lavage and endoscopy, highlighting that these diagnostic modalities can and should work in tandem for the larger goal of preventing the development of carrier animals and preventing future outbreaks.

4.3 Bacteriology of the guttural pouch

The guttural pouch has been shown to be an incredibly dynamic environment, much more than previously thought, with a complex interplay between the bacteria and host occurring as *S. equi* infection becomes persistent. The structural variants observed in the third chapter were located in genomic regions encoding and surrounding the hyaluronic acid capsule and key sortase-processed cell surface-anchored proteins. Deletions and inversions could provide survival advantages in the guttural pouch of a carrier animal, hence their presence in isolates recovered from animals with persistent infection. The effects are unknown but are likely to benefit persistence within the guttural pouch, with the hyaluronic acid capsule being linked to immune evasion and adherence and sortase-processed cell surface-anchored proteins being linked to colonisation and virulence. In the second chapter, the dual-target iELISA was shown to be unable to detect carrier animals, it is possible that the structural variants observed in the third chapter and elsewhere (Chanter *et al.*, 2000, Harris *et al.*, 2015) could explain this failure.

The complexity of the guttural pouch environment is emphasised by the findings of the second chapter; when the strangles-screening process at a UK rescue centre was evaluated, it was found that 28.9% (n=11/38) of equids with strangles tested positive for *S. zooepidemicus* and negative for *S. equi*. The role of *S. zooepidemicus* in these cases is unclear and this finding contributes to the complex nature of persistence presented. Inversion sequences also present the possibility of subpopulations coexisting within the guttural pouch environment. It is not known whether *S. zooepidemicus* was acting as a primary or secondary pathogen, whether it had a greater propensity to cause chondroids or if it colonised after the chondroids had formed and whether it presented a true infective risk to other equids. It may be that *S. equi* infection benefits *S. zooepidemicus*, providing the environment and opportunity for colonisation at the expense of its own persistence. The structural variants that result in bacteria becoming suited to persistence, including metabolic streamlining and the loss of virulence factors, could render *S. equi* less adaptable to changing environments and more susceptible to *S. zooepidemicus* colonisation. *S. zooepidemicus* is a less pathogenic bacteria than *S. equi* but is more flexible and can survive in a greater range of environments. The specialisation of *S. equi* to the guttural pouch, and

further specialisation as the animal enters the carrier state, may have consequences for the interaction of these bacteria and their subpopulations.

4.4 Recommendations for future research

Understanding *S. equi* is crucial to combatting strangles and much work has been carried out to characterise its evolution (Holden *et al.*, 2009), genome (Harris *et al.*, 2015), epidemiology (Mitchell *et al.*, 2021), survivability (Durham *et al.*, 2018), resistance profile (Fonseca *et al.*, 2020) and pathogenicity (Timoney and Kumar, 2008, Timoney, 2004). This increased understanding has enabled the development of more targeted diagnostic assays (Noll *et al.*, 2020, Webb *et al.*, 2013, Willis *et al.*, 2021, Boyle *et al.*, 2021), better outbreak prevention and management protocols (Rendle *et al.*, 2021) and a safe and efficacious vaccine with DIVA capability (Robinson *et al.*, 2020); these advances better equip clinicians and caregivers to treat and prevent strangles.

Characterising the bacterial populations and subpopulations present within the guttural pouch environment is crucial to understanding equine strangles and bacterial persistence. Clinical guidelines, diagnostic tests and vaccines are informed by this knowledge and the nuanced picture presented has implications for clinicians and caregivers. Following from the findings of this study, future research directions should include:

- Investigation into the wider microbiome of the guttural pouch, including the dynamics and potential interactions between *S. equi* and other bacteria, could provide important insights into the development and persistence of strangles. There has been little examination of the makeup and impact of the broader microbiome in this environment.
- Further research with a greater number of more representative samples into the possible subpopulations of *S. equi* within the guttural pouch and their interactions with each other would yield valuable information regarding the prevalence and impact of inversion sequences.
- Studying the impact of the structural variants found in this study on the transcriptome, methylome, and proteome of *S. equi* could yield important insights into the effects of deletions, insertions and inversions and their role in the pathogenesis and persistence of *S. equi*. In addition, assessing hyaluronic acid capsule depth in growth media would provide a relatively easy way to assess the impacts of deletions and insertions in the *has* locus.

- Further research is needed to determine whether this Javan prophage, found to be integrated within genes encoding the hyaluronic acid capsule, is linked to increased (or decreased) virulence and pathogenicity. Additional sequence analysis may identify more phage in *S. equi* and reveal information of their prevalence and impact.
- The potential impact of structural variants on commercially available diagnostic tests and vaccines should also be explored. This could be done by sequencing strains of interest from animals where full clinical information is available, and determining whether the outcomes diagnostic tests correlate with the presence or absence of particular structural variants.
- Investigating the relationship between quantitative PCR CT values and infectivity would aid in the management of equine strangles as the infective risk is currently unclear for equids with a high CT value.
- More research is needed to determine the role of *S. zooepidemicus* in equine strangles and related infections, including its potential as a primary or secondary pathogen.
- Further research is required to determine what circumstances are necessary for *S. zooepidemicus* to colonise and cause strangles infections in equids, and whether they should be managed the same as *S. equi* infections. Sequence analysis of *S. zooepidemicus* samples from in-contact cases could help identify whether transmission occurs between horses.

4.5 Conclusion

Strangles is a persistent threat to equid health and will continue to be for the foreseeable future. Each advance in research reveals insight into the detection and management of this persistent disease. Analysis of the genome of *S. equi* can reveal insight, as in the case of this study, but there is still much to be studied and understood. Likewise, clinical research builds the evidence to recommend gold-standard diagnostics and treatments to those working in the field.

Extensive research throughout the last 30 years has ensured that this disease is now well-understood, treatable, and, crucially, preventable. This study showed that well-constructed screening protocols, with appropriate quarantining alongside guttural pouch lavage and endoscopy, represents an effective avenue of strangles control; prevention of this disease will always be preferable to retrospective management. However, economic, and practical factors inevitably prevent their uptake in many equestrian facilities. When screening is not possible, vaccination could ensure equids have an immune response that can robustly combat this ancient disease. This project benefitted from the pursuit of two different research strands with one focusing on the host and the other on the analysis of pathogen's genome. When overlap was present, novel insights were revealed, for example the structural variants found in the genomic analysis could help explain the failure of the dual-target iELISA test. Each strand of the project had benefits and limitations, as discussed in the relevant chapters, but by taking this approach greater insight was gained about both host and molecular mechanisms contributing to the persistence of equine strangles.

Studies such as this help unravel the complexities of strangles so that clinicians and caregivers have the tools to diagnose, treat, control, and prevent equine strangles. There is undoubtedly more work to be done, but it is evident that eradication is now possible for this endemic disease that was once considered an inevitability.

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6 Appendix

6.1 Table of genes investigated

Table 6.*:* The genes investigated in JKS063a, replicated in all isolates, using SE4047 as a reference.

Gene: SE4047	Gene: JKS063a	Product	Strand	Orientation
SeM protein: SEQ_2011 to SEQ_2022				
SEQ_2011	SEE_19540	glucan 1%2C6-alpha-glucosidase	Reverse	0
SEQ_2012	SEE_19550	multiple sugar-binding transport ATP-binding protein	Reverse	0
SEQ_2013	SEE_19560	leucine-rich protein	Reverse	0
skc	skc	streptokinase	Forward	1
SEQ_2015	SEE_19580	D-tyrosyl-tRNA(Tyr) deacylase	Reverse	0
SEQ_2016	SEE_19590	GTP pyrophosphokinase	Reverse	0
fbp	fbp	antiphagocytic cell surface-anchored fibrinogen-and IgG Fc-binding protein SeM	Reverse	0
SEQ_2018	SEE_19610	AraC family regulatory protein	Forward	1
SEQ_2019	SEE_19620	putative NrdI-like protein	Forward	1
SEQ_2020	SEE_19630	endonuclease/exonuclease/phosphatase family protein	Reverse	0
SEQ_2021	SEE_19640	putative glucose-specific phosphotransferase system (PTS)%2C IIABC component	Reverse	0
SEQ_2022	SEE_19650	LacI family regulatory protein	Reverse	0
Sortase-processed surface proteins: SEQ_2180 to SEQ_2190				
SEQ_2179	SEE_21180	GTP pyrophosphokinase	Reverse	0
SEQ_2180	SEE_21190	putative cell surface-anchored protein	Forward	1
SEQ_2181	SEE_21200	putative sensor kinase	Forward	1
SEQ_2182	SEE_21210	putative response regulator	Forward	1
SEQ_2183	SEE_21220	Pseudomonas avirulence D protein-like protein	Forward	1
SEQ_2184	SEE_21230	hypothetical protein	Forward	1
SEQ_2185	SEE_21240	putative transposase	Reverse	0
SEQ_2186	SEE_21250	putative transposase	Reverse	0
SEQ_2187	SEE_21260	hypothetical protein	Forward	1
SEQ_2188	SEE_21270	ABC transporter%2C ATP-binding protein	Forward	1
SEQ_2189	SEE_21280	putative permease protein	Forward	1
SEQ_2190	SEE_21290	putative cell surface-anchored protein	Forward	1
SEQ_2191	SEE_21300	putative membrane protein	Forward	1
Sortase-processed surface proteins: SEQ_0932 to SEQ_0945				
SEQ_0932	SEE_09100	ABC transporter ATP-binding protein	Forward	1
SEQ_0933	SEE_09110	fibrinogen-binding cell surface-anchored protein SzPSe	Forward	1
SEQ_0934	SEE_09120	hypothetical protein	Reverse	0
SEQ_0934	SEE_09130	hypothetical protein	Reverse	0
SEQ_0935	SEE_09140	collagen binding%2C putative ancillary pilus subunit Cne	Forward	1
SEQ_0936	SEE_09150	putative backbone pilus subunit (T6-antigen-like)	Forward	1
SEQ_0937	SEE_09160	sortase SrtC1	Forward	1
SEQ_0938	SEE_09170	putative Mac family protein	Forward	1
SEQ_0939	SEE_09180	putative cell surface-anchored protein	Forward	1
SEQ_0940	SEE_09190	hypothetical protein	Reverse	0
SEQ_0941	SEE_09200	hypothetical protein	Reverse	0
SEQ_0942	SEE_09210	hypothetical protein	Reverse	0
SEQ_0943	SEE_09220	hypothetical protein	Reverse	0
SEQ_0944	SEE_09230	putative cell surface-anchored pullulanase	Forward	1
Has: SEQ_0260 to SEQ_0274				
SEQ_0264	SEE_02230	BioY family protein	Forward	1
SEQ_0265	SEE_02240	metallo-beta-lactamase superfamily protein	Forward	1
SEQ_0266	SEE_02250	putative deaminase	Forward	1
SEQ_0267	SEE_02260	putative lipoprotein	Reverse	0

SEQ_0268	SEE_02810	putative membrane protein	Forward	1
hasA	SEE_02820	hyaluronan synthase	Forward	1
hasB	SEE_02830	UDP-glucose 6-dehydrogenase	Forward	1
hasC1	hasC1	UTP--glucose-1-phosphate uridylyltransferase 1	Forward	1
SEQ_0273	SEE_02850	hypothetical protein	Reverse	0
SEQ_0274	SEE_02860	5-formyltetrahydrofolate cyclo-ligase family protein	Reverse	0
Equibactin: SEQ_1231 to SEQ_1249				
SEQ_1231	SEE_05290	putative DNA-binding protein	Reverse	0
SEQ_1232	SEE_05300	hypothetical protein	Reverse	0
eqbN (-)	eqbN	putative hydrolase	Reverse	0
eqbM (-)	eqbM	putative oxidoreductase	Reverse	0
eqbL (-)	eqbL	ABC transporter%2C ATP-binding membrane protein	Reverse	0
eqbK (-)	eqbK	ABC transporter%2C ATP-binding membrane protein	Reverse	0
eqbJ (-)	eqbJ	ABC transporter%2C ATP-binding component	Reverse	0
eqbI (-)	eqbI	ABC transporter permease protein	Reverse	0
eqbH (-)	eqbH	ABC transporter permease protein	Reverse	0
eqbG (-)	eqbG	equibactin nonribosomal peptide synthase protein	Reverse	0
eqbF (-)	eqbF	putative thiazoline reductase	Reverse	0
eqbE (-)	eqbE	equibactin nonribosomal peptide synthase protein	Reverse	0
eqbD (-)	eqbD	putative salicylate-AMP-ligase	Reverse	0
eqbC (-)	eqbC	putative 4'-phosphopantetheinyl transferase	Reverse	0
eqbB (-)	eqbB	putative non-ribosomal peptide synthesis thioesterase type II	Reverse	0
eqbA (-)	eqbA	iron-dependent repressor protein	Reverse	0
SEQ_1247	SEE_05450	putative conjugative transposon membrane protein	Reverse	0
SEQ_1249	SEE_05460	putative conjugative transposon mobilization protein	Forward	1
MutX: SEQ_1411 to SEQ_1420				
SEQ_1411	SEE_07050	ABC transporter permease protein	Reverse	0
SEQ_1412	SEE_07060	putative exported protein	Reverse	0
SEQ_1413	SEE_07070	putative fibronectin/fibrinogen-binding protein	Forward	1
SEQ_1414	SEE_07080	hypothetical protein	Reverse	0
SEQ_1415	SEE_07090	putative membrane protein	Reverse	0
SEQ_1416	SEE_07100	mutator MutX protein (7%2C8-dihydro-8-oxoguanine-triphosphatase)	Reverse	0
SEQ_1417	SEE_07110	putative dTDP-glucose-4%2C6-dehydratase	Reverse	0
SEQ_1418	SEE_07120	putative dTDP-4-keto-6-deoxyglucose-3%2C5-epimerase	Reverse	0
SEQ_1419	SEE_07130	glucose-1-phosphate thymidyl transferase	Reverse	0
SEQ_1420	SEE_07140	FAD dependent oxidoreductase	Reverse	0
Citrate: SEQ_1197 to SEQ_1211				
SEQ_1197	SEE_00300	putative carboxylase subunit	Forward	1
SEQ_1198	SEE_00290	putative puatative apo-citrate lyase phosphoribosyl-dephospho-CoA transferase	Forward	1
SEQ_1199	SEE_00280	putative citrate lyase alpha subunit	Forward	1
SEQ_1200	SEE_00270	putative citrate lyase beta subunit	Forward	1
SEQ_1201	SEE_00260	putative citrate lyase acyl carrier protein	Forward	1
SEQ_1202	SEE_00250	putative membrane protein	Forward	1
SEQ_1203	SEE_00240	putative Na+-transporting methylmalonyl-CoA/oxaloacetate decarboxylase%2C beta subunit	Forward	1
SEQ_1204	SEE_00230	putative Na+-transporting methylmalonyl-CoA/oxaloacetate decarboxylase%2C beta subunit	Forward	1
SEQ_1205	SEE_00220	hypothetical protein	Forward	1
SEQ_1205	SEE_00210	hypothetical protein	Forward	1
SEQ_1206	SEE_00200	putative Mg2+/citrate complex transporter	Reverse	0
SEQ_1207	SEE_00190	GntR family regulatory protein	Reverse	0
SEQ_1208	SEE_00180	putative 2-(5''-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase	Reverse	0
SEQ_1209	SEE_00170	hypothetical protein	Forward	1
SEQ_1210	SEE_00160	hypothetical protein	Forward	1

SEQ_1211	SEE_00150	putative Citrate [pro-3S]-lyase] ligase	Reverse	0

6.2 Skills and training record

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

School of Veterinary Medicine and Science

Skills and continual professional development training portfolio

This record is to be maintained by the postgraduate student who will retain it. It is to be used by supervisors and internal assessor at the Annual Review.

STUDENT DETAILS			
Name of Student	Luke McLinden	Start Date:	01/05/2023
Student ID	20143214	Current year of registration:	2022/2023
Main Supervisor	Sarah Freeman		

SKILLS PORTFOLIO

The level of competency is SELF ASSESSED and rated on a scale of 1 to 5: 1 = Unsatisfactory, 2 = Needs development, 3 = Adequate, 4 = Proficient, 5 = Competent

Skill Description	Level of Competency					Date	Comments
	1	2	3	4	5		
Critique of scientific papers			x			15/01/2023	I am adequate at the critical appraisal of papers, but sometimes require prompts as to what I should be focusing on. I am working on this and improving as I write my discussion.
Use of reference manager				x		11/08/2022	I have appropriately used a reference manager in my literature review but need to better organise my references within the library. I feel confident in my use of reference manager.
Communication skills				x		16/02/2023	I am happy with my ability to communicate in a several different mediums (e.g., written, verbal), but need to develop my ability to adapt communication

							style dependent on targeted audience (e.g scientific, non- scientific.)
Team working abilities					x	08/03/2023	I have consistently demonstrated my ability to work in team in a clinical, laboratory and teaching environment. I feel I work effectively and efficiently as a team worker.
Practical skills of lab, or other, methods			x			16/02/2023	I have been working in the lab for several months and have consistently struggled with many elements. I have been able to carry out the work, but this area of my skillset requires further development.
Poster design and presentation			x			27/07/2022	I initially struggled with art and design but have been able to produce a poster for the AVMA presentation and create a figure for my literature review.
Verbal presentation				x		05/12/2022	I have presented my research a number of times over the past few months and have received positive feedback. Overall, I feel confident in my verbal presentation skills as a result of these experiences. I need to work on tailoring the content for my audience.
Written presentation				x		22/10/2023	I have struggled with presenting my work in the past but have found this to be much easier as I have improved my digital literacy by attending university led courses. I am now comfortable with my ability to present written work.
Writing skills					x	22/10/2023	In many ways, I feel this is my strongest skill as I am confident in my ability to write proficiently and quickly in a scientific manner.
Clinical skills (if applicable)				x		30/12/2022	I have become proficient at performing guttural pouch lavage and endoscopy as a result of my time at Bransby Horses and the Defense Animal Centre.

This record is to be maintained by the Postgraduate Student. It is to be submitted each year to accompany the written report, as part of the Annual Review process.

I declare that I have undertaken mandatory research integrity training ☒ Date: 01/02/2023

List of Courses/Presentations/Conferences/Seminars/Scientific Meetings etc.	Date Attended	Credits	Cumulative credits
Central university course whole day (Microsoft word – managing long docs)	20/06/2022	2	2
Central university course whole day (Microsoft excel – sort, filter and pivot data)	17/10/2022	2	4
Facilitation training	07/06/2022	2	6
Demonstrator training	18/05/2022	2	8
Facilitated at multistakeholder symposium on equine euthanasia	24/05/2022	2	10
AVMA poster presentation	12/05/2022	2	12

SGTR clinical relevance facilitation: 10 sessions (1hr each)	06/2022 – 11/2022	10	22
Undergraduate demonstrator (AHW): 9 sessions (3hrs each)	07/2022 – 03/2023	18	40
Undergraduate demonstrator (GIL): 5 sessions (4hrs each)	05/2022 - 12/2022	20	60
Undergraduate demonstrator (NMSK): 4 sessions (4hrs each)	06/2022 – 01/2023	16	76
Undergraduate demonstrator (ENI): 4 sessions (4 hrs each)	07/2022 – 01/2023	16	92
Presented research findings to the equine research group and Bransby welfare centre	28/11/2022	4	96
Presented research findings at the postgraduate winter symposium	05/12/2022	2	98