Streptococcus equi: a pathogen restricted to one host

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Strangles caused by the host adapted Lancefield group C Streptococcus equi subspecies equi (S. equi) is a frequently diagnosed infectious disease of horses worldwide. Critical to the global success of S. equi is its ability to establish persistent infections within the guttural pouches of recovered apparently healthy horses that can result in transmission to in-contact animals. Recent research has identified key events in the S. equi genome, which occurred during its evolution from an ancestral strain of S. equi subspecies zooepidemicus, that may enhance its ability to evade host innate immune responses and rapidly multiply in the tonsillar complex and draining lymph nodes. This review discusses the role of these genetic events on the evolution and emergence of this important host-restricted pathogen.

Introduction

Strangles caused by the bacterium Streptococcus equi subspecies equi (S. equi) continues to be the most frequently diagnosed infectious disease of horses worldwide. This highly contagious disease is characterized by abcessation of lymph nodes in the head and neck, and leads to high morbidity and occasional mortality (Timoney, 1993). Extrapolation based on figures in the Equine Quarterly Disease Surveillance Reports (http://www.aht.org.uk/equine_disease.html) and data from a recent study of UK strangles outbreaks (Ivens et al., 2011) suggests there were over 700 outbreaks of strangles in the UK during 2008. Some of these outbreaks involved over 200 horses, and led to significant economic and welfare costs.

The worldwide incidence of strangles is unexpectedly high given that the acute infection should be readily identified, contained and resolved using standard biosecurity measures. However, a proportion of recovered horses fail to drain pus from their guttural pouches following rupture of abscesses formed in adjacent retropharyngeal lymph nodes. Over time this pus becomes inspissated to form ‘chondroids’, enabling S. equi to persist for up to several years and transmit to naïve horses (Newton et al., 2000). The global success of S. equi has likely stemmed from its ability to rapidly proliferate in tonsil tissue and draining lymph nodes, subsequently leading to abscesses that drain into the guttural pouches where infection persists. The selective requirements of these very different host environments have likely influenced the events of gene loss and gain that have shaped the S. equi genome.

S. equi is believed to have evolved from an ancestral strain of S. equi subspecies zooepidemicus (S. zooepidemicus) (Jorm et al., 1994; Webb et al., 2008), which is associated with a wide variety of diseases in horses and other animals including humans. Both of these organisms share over 80% DNA sequence identity with the important human pathogen Streptococcus pyogenes. S. equi in particular has much in common with S. pyogenes; both are host-restricted pathogens, share near identical surface proteins, phospholipase A₂ and superantigens (sAgs), and have acquired the genes encoding these toxins as cargo on related prophage suggesting that they share a common phage pool enabling cross-species gene transfer (Holden et al., 2009). Both pathogens also cause diseases that share similarities with one another, strangles and tonsillitis, and so the study of S. equi may shed new light on S. pyogenes infections of humans.

S. equi, a poor colonizer

S. zooepidemicus usually infects young horses by entering the nose or mouth and colonizes the mucosal surface and tonsillar tissues of the nasopharynx. S. equi also infects via the nose or mouth, most likely through contaminated drinking water, but does not colonize the nasopharynx and is often not detected by nasopharyngeal swabs or washes taken 24 h post-infection. Interestingly, unlike S. zooepidemicus, S. equi does not persist in the tonsils and only rarely infects other animal species (Ladlow et al., 2006) including humans (Breiman & Silverblatt, 1986).

Multilocus sequence typing of 253 isolates of S. zooepidemicus and 24 isolates of S. equi revealed that S. equi is most closely related to a cluster of S. zooepidemicus strains that were significantly associated with isolation from cases of uterine infection or abortion rather than the upper...
Interestingly, the SeP9 bacteriophage, which is lysogenic in and shown to bind collagen (Lannergård locus is present in the genomes of every strain of S. equi strain 4047, isolated from the equine upper respiratory tract, encodes 39 sortase-processed proteins putatively attached to the cell wall by virtue of a C-terminal LPxTG motif. In contrast, S. equi strain 4047 encodes 29 such proteins and lacks functional homologues of several S. zooepidemicus sortase-processed cell surface proteins, including FimII, FimIII and FimIV pilus loci (Beres et al., 2008; Holden et al., 2009). Pili play an important role in the adherence of streptococci to host tissues (Abbot et al., 2007; Pointon et al., 2010), and the reduced complement of pili and other sortase-processed proteins on the surface of S. equi may limit its ability to bind to an array of host tissues and to occupy diverse pathogenic niches.

Although the number of putative sortase-processed cell surface proteins is reduced in S. equi, it retains the FimI locus, whose accessory pilin, CNE, shares homology to CNA of Staphylococcus aureus and has been similarly shown to bind collagen (Lannergård et al., 2003). The gene encoding the putative TetR-like repressor of the FimI locus of S. equi was found to contain a nonsense mutation at codon 43, which hypothetically would lead to deregulation and constitutive or elongated pilus production (Holden et al., 2009; Swierczynski & Ton-That, 2006). This pilus locus is present in the genomes of every strain of S. equi and S. zooepidemicus examined to date, as determined by PCR (Lannergård et al., 2003), quantitative PCR or genome sequencing (K. Steward, unpublished data), and is likely to play an important role in general adhesion to host tissues. Such a function may enable S. equi to attach to tonsillar epithelium. In support of this, a new multicomponent subunit vaccine containing recombinant CNE conferred protection against S. equi infection in Welsh mountain ponies (Guss et al., 2009).

In contrast to the majority of S. zooepidemicus strains, S. equi lacks the ability to ferment ribose, sorbitol and lactose as a consequence of independent lesions in its genome (Holden et al., 2009). This difference in metabolism is commonly used to differentiate cultures of S. equi from S. zooepidemicus (Bannister et al., 1985). Utilization of different carbohydrates plays an important role in the ability of streptococci to colonize mucosal surfaces (Shelburne et al., 2008), and reduction of its metabolic capabilities could further reduce the potential of S. equi to persist on the surface and in the crypts of the equine tonsil.

S. equi does not possess a clustered regularly interspaced short palindromic repeat (CRISPR) locus, which confers resistance to phage attack and invading DNA (Barrangou et al., 2007; Holden et al., 2009; Sorek et al., 2008). Interestingly, the SeP9 bacteriophage, which is lysogenic in S. equi, does not replicate in S. zooepidemicus (Tiwari & Timoney, 2009). S. equi also lacks several putative competence loci, whose presence may be incompatible with lysogeny (Beres et al., 2008; Holden et al., 2009). Consequently, S. equi is polylysogenic and temperate phage is easily demonstrated (Spanier & Timoney, 1977). The strain 4047 genome contains four different prophages, which may each contribute to the adaptation of S. equi to its unique pathogenic niche (Holden et al., 2009). Unlike the other three prophage, ϕSeq1 contains no ‘cargo’ genes and was induced with mitomycin C. Interestingly, the sixth CRISPR spacer of S. zooepidemicus strain H70 exactly matches part of SEQ0163 of ϕSeq1, suggesting that this strain of S. zooepidemicus has previously been attacked by a ϕSeq1-like prophage (Holden et al., 2009).

The cargo of ϕSeq2 encodes a putative phospholipase A2 with 98 % predicted amino acid sequence identity with SlaA of S. pyogenes M3 strain MGAS315. The acquisition of slaA by S. pyogenes M3 was associated with increased morbidity and mortality in humans (Brüssow et al., 2004). Deletion of slaA reduced the ability of S. pyogenes to colonize the upper respiratory tract of a macaque model of pharyngitis and reduced its virulence in a mouse intraperitoneal infection model (Sitkiewicz et al., 2006). SlaA was present in 31 % of a diverse population of S. zooepidemicus strains, suggesting that this toxin may also be important to this opportunistic pathogen (Holden et al., 2009). A second gene encoding a putative phospholipase A2 toxin, SlaB, sharing 70 % amino acid sequence identity with SlaA and associated with a phage remnant, suggesting horizontal acquisition, was identified in all strains of S. equi and S. zooepidemicus examined by sequencing or quantitative PCR (Holden et al., 2009). The contribution of these toxins to both S. equi and S. zooepidemicus virulence is unknown (Fig. 1).

### Invasion and abscessation of lymph nodes

Rather than colonizing the epithelial surface, S. equi quickly invades tonsillar tissue where it multiplies rapidly producing many extracellular microcolonies. It has been detected in the lymph nodes of the head and neck within as little as 3 h post-infection (Timoney & Kumar, 2008). An array of factors are produced that putatively enhance its ability to replicate in vivo and misdirect the equine immune response.

Acquisition of iron is an essential process for all pathogenic bacteria (Brown & Holden, 2002). In mammalian hosts tissue iron is sequestered by transferrin, lactoferrin, or inside red blood cells (Wooldridge & Williams, 1993). Bacterial pathogens access this limited iron supply through the production of cell surface receptors for transferrin and lactoferrin, utilizing haem-containing compounds, iron transporters, and the synthesis and secretion of iron-sequestering siderophores (Wandersman & Delepaire, 2004).

A unique S. equi iron-capture mechanism that is absent from all S. zooepidemicus strains examined to date is encoded.
by a novel integrative conjugative element ICESe2 (Holden et al., 2009). The cargo region of this ICE encodes proteins sharing similarity with the non-ribosomal peptide synthesis (NRPS) system of Clostridium kluveri and Yersinia sp. that produce an unnamed siderophore (Seedorf et al., 2008) and the ferric iron-binding siderophore yersiniabactin (Bobrov et al., 2002), respectively. The S. equi NRPS system produces an as yet undefined secreted molecule, provisionally named equibactin, which enhances the ability of S. equi to acquire iron in vitro (Heather et al., 2008) (Fig. 2). Siderophore biosynthesis has not previously been identified in any Streptococcus sp. (Eichenbaum et al., 1996), but plays an important role in the virulence of many important pathogens (Brown & Holden, 2002), including Yersinia pestis, the causative agent of bubonic plague (Bearden et al., 1997). Acquisition of ICESe2 may increase the ability of S. equi to acquire iron in vivo, and enhance its ability to proliferate in tonsil and lymph node tissue.

The S. equi and S. zooepidemicus genomes also encode an HtsABC haem-binding system (Nygaard et al., 2006), a putative MsABC Mn²⁺ and Fe³⁺ metal transport system with 80 to 91 % amino acid sequence identity to that of S. pyogenes (Janulczyk et al., 1999) and a putative FtsABCDFe³⁺ ferrichrome transport system with 59 to 77 % amino acid sequence identity to that of S. pyogenes (Hanks et al., 2005). Although a recent report suggested that the HtsABC system is functional in S. equi (Nygaard et al., 2006), the gene in the S. equi strain 4047 genome encoding the first haem-binding protein of this system, Shr (Zhu et al., 2008), contains a frameshift mutation at codon 442 that truncates this protein (Holden et al., 2009) resulting in its secretion. Secreted Shr lacks haem- or fibronectin-, but retains haemoglobin- and haemoglobin–haptoglobin-binding capabilities (Meehan et al., 2010). Truncation of Shr either has an as yet unknown functional benefit for S. equi, or reflects redundancy in iron-uptake mechanisms following gain of the equibactin NRPS system.

S. equi and S. zooepidemicus produce streptolysin S (SLS), which is responsible for the characteristic zone of beta-haemolysis surrounding colonies on blood agar plates (Flanagan et al., 1998). The toxin is almost identical to that produced by most strains of S. pyogenes, which has a direct cytopathic effect on many host cell types (Wannamaker, 1983). S. pyogenes also produces a streptolysin O (SLO), which is not made by S. equi or S. zooepidemicus (Flanagan et al., 1998). Deletion of SLO or SLS alone or in combination has shown that each contributes to the early stages of S. pyogenes infection in a subcutaneous mouse infection model (Fontaine et al., 2003). In agreement with these results, deletion of sagA, encoding SLS in S. equi strain 4047 abolished beta-haemolytic activity in vitro and delayed the onset of clinical signs in a murine intranasal infection model by 24 h (C. Robinson, unpublished data). These data suggest that SLS plays a role in S. equi virulence, but indicate that other factors are also important to the ability of this organism to cause disease.
Two other prophage of *S. equi* strain 4047 encode four bacterial sAgs – SeeL and SeeM encoded on *φ*Seq3, and SeeH and SeeL encoded on *φ*Seq4, which share homology with the mitogenic toxins of *S. pyogenes* (Artiushin et al., 2002; Paillot et al., 2010b; Proft & Fraser, 2003; Proft et al., 2003). sAgs are potent immuno-stimulatory molecules that disrupt innate and adaptive immune responses through non-specific T lymphocyte proliferation and the generation of an overzealous pro-inflammatory response (Llewelyn & Cohen, 2002; Sriskandan et al., 2007). sAg activities are based on their ability to bypass the mechanism of MHC-restricted antigen presentation (Dellabona et al., 1990). Conventional exogenous antigens are processed and presented by antigen presenting cells within the antigen groove of specific MHC class II molecules and recognized by antigen-specific TCRs (T-cell receptors), which results in highly specific T-cell activation (1 out of $1 \times 10^6$ T lymphocytes activated). Secreted sAgs bind as intact proteins directly to the MHC class II molecule outside the peptide-binding site and to one or more specific TCR Vβ chains leading to the activation of 5–20% of the T-cell population (Li et al., 1999; Llewelyn & Cohen, 2002) (Fig. 3).

SeeL, SeeL and SeeM of *S. equi* have been shown to stimulate proliferation of equine peripheral blood mononucleated cells (PBMC) *in vitro* (Anzai et al., 1999a; Artiushin et al., 2002; Paillot et al., 2010b) (Fig. 4). A febrile response in ponies was elicited by SeeL but not by SeeH. There is also some discrepancy in the level of mitogenic activity induced by SeeH *in vitro*, which was active on equine PBMCs in one study (Artiushin et al., 2002), but active only on asinine PBMCs in another (Paillot et al., 2010b). Such differences might be explained by variation in the affinity of sAgs for different MHC class II and TCR molecules. HLA class II polymorphisms are known to influence the nature of T-cell responses to *S. pyogenes* sAgs (Llewelyn et al., 2004) and the risk of severe streptococcal infection in humans (Kotb et al., 2002). The recently sequenced equine genome will provide the opportunity to identify equine MHC class II molecules recognized by *S. equi* sAgs and quantify the risks associated with the production of particular alleles thereof (Wade et al., 2009). Recent sequencing of *S. zooepidemicus* strain BHS5 identified three new sAg-encoding genes: *szeF*, *szeN* and *szeP*, the presence of which was significantly higher in isolates from non-strangles lymph node abscesses (Paillot et al., 2010a), suggesting that sAgs play an important role in the ability of these isolates to cause disease in lymph nodes.

The truncated fibronectin-binding protein, FNE, of *S. equi* lacks its C-terminal cell wall sorting LPxTG motif resulting in its secretion (Lindmark et al., 2001). However, despite this truncation secreted FNE maintained its fibronectin- and collagen-binding activity enabling the induction of cell-mediated collagen gel contraction by linking fibronectin to collagen type-I fibres and normalization of interstitial fluid pressure (Lidén et al., 2008). Acute strangles is often accompanied by oedema on the afferent side of infected mandibular and suprapharyngeal nodes, which may be an effect of contraction of collagen in the capsule of these nodes.

One consequence of the acute phase inappropriate immune response generated by *S. equi* is a rise in body temperature.

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**Fig. 3.** The prophages *φ*Seq3 and *φ*Seq4 contain the ‘cargo’ genes *seeL*/*seeM* and *seeH*/*seeL*, respectively. *S. equi* sAgs interact with the equid MHC class II molecules and TCR, bypassing the mechanism of MHC-restricted antigen presentation.
Artiushin et al. (2002), which can be monitored and detected in the absence of bacterial shedding. Therefore, once a strangles outbreak is confirmed, presumptively infected pyrexic horses may be identified and isolated before the organism is passed to in contact animals.

Resistance to phagocytosis

*S. equi* deploys an array of anti-phagocytic factors and mechanisms that frustrate and confound the innate immune response. In common with many other pathogenic bacteria, *S. equi* produces a hyaluronic acid capsule that mimics the molecule in vertebrate tissue and shields the bacterium from immune recognition (Woolcock, 1974). Although it appears that strains producing different levels of capsule *in vitro* retain the same ability to infect horses (Anzai et al., 1999b) either because the multitude of anti-phagocytic mechanisms exploited by *S. equi* confer a level of redundancy or because other factors expressed by strains may compensate. The high level of capsule production in *S. equi* relative to many *S. zooepidemicus* strains (Fig. 5) may reflect differences in operon structure (Blank et al., 2008; Holden et al., 2009) that affect biosynthesis, or differences in hyaluronic acid turnover (Holden et al., 2009). *S. equi* encodes and produces a non-secreted hyaluronate lyase on *φSeq4* with low activity (Lindsay et al., 2009), but lacks a functional copy of the genome-encoded hyaluronate lyase (Holden et al., 2009), whose orthologues have a broader substrate range and greater activity (Pritchard et al., 1994). Reduced hyaluronic acid and chondroitin turnover may explain why *S. equi* infection rarely spreads beyond the lymphatic system and why the majority of horses recover from strangles despite possessing extremely high bacterial loads during the acute phase of disease.

The anti-phagocytic surface protein SeM binds fibrinogen, and IgG4 and IgG7 subclasses (Lewis et al., 2008; Meehan et al., 2001). Fibrinogen binding masks C3b-binding sites on the bacterial surface reducing the rate of phagocytosis (Boschwitz & Timoney, 1994; Lewis et al., 2008; Meehan et al., 2001). The C-terminal two-thirds of SeM is predicted to have an alpha-helical coiled-coil structure and shares close sequence similarity with SzM of *S. zooepidemicus* (Kelly et al., 2006) whereas, the N-terminal third is predicted to possess non-coiled-coil single strands and is unique to *S. equi* (Timoney et al., 1997). SeM binds fibrinogen through residues located at the extreme N terminus of SeM, but requires the stabilizing coiled-coil structure (Meehan et al., 2000). In contrast, the IgG-binding site of SeM is located in the central region (amino acid residues 273–357) between the A and B repeats (Meehan et al., 2001). Studies utilizing recombinant SeM proteins containing defined internal deletions and protein chimeras consisting of the non-IgG-binding *S. pyogenes* M5 protein fused to SeM sequences identified a region of 14 amino acids (335–348) that was critical for IgG, but not fibrinogen binding (Meehan et al., 2009). It was also noted that binding of IgG to SeM did not reduce its ability to bind fibrinogen.
Se18.9 binds factor H and inhibits phagocytosis by reducing C3 deposition on the bacterial surface (Tiwari et al., 2007). Se18.9 was produced by all 26 strains of S. equi examined, but by only 1 of 140 strains of S. zooepidemicus (Holden et al., 2009), suggesting that this gene could have been important to the evolution of S. equi.

S. equi encodes two different IgG endopeptidases – IdeE and IdeE2 (Hulting et al., 2009; Lannergård & Guss, 2006) – which share amino acid sequence identity with IdeZ and IdeZ2 of S. zooepidemicus and IdeS/Mac IgG endopeptidase of S. pyogenes (Lei et al., 2001; Von Pawel-Rammingen et al., 2002). These enzymes cleave IgG produced by a variety of animal species, thereby reducing IgG recognition and targeting of bacteria by the immune response. IdeE2 and IdeZ2 cleave equine IgG more efficiently than IdeE and IdeZ (Hulting et al., 2009), but there remains some uncertainty as to whether the main antiphagocytic mechanism exploited by IdeE is reduction of opsonophagocytosis via IgG cleavage (Liu & Lei, 2010; Timoney et al., 2008). Rather, its dose dependent inhibitory effect on the bactericidal activity of neutrophils for both S. equi and S. zooepidemicus, which is neutralized by a specific antibody response, suggests a different mechanism of reaction. Interestingly, the inclusion of recombinant IdeE and IdeE2 significantly enhanced the efficacy of a multi-component subunit vaccine against strangles, suggesting that antibodies that target these secreted proteins are important for the development of protective immunity against S. equi in the horse (Guss et al., 2009).

Interference with neutrophil recruitment theoretically should enhance the ability of S. equi to evade immune clearance. S. equi encodes a sortase-processed cell envelope proteinase (SeCEP) with 59% amino acid identity to SpyCEP of S. pyogenes (Turner et al., 2009), which cleaves and inactivates interleukin 8 and other CXC chemokines (Edwards et al., 2005; Turner et al., 2009). Heterologous production of SpyCEP in Lactococcus lactis transformed this normally avirulent organism into one that caused rapid onset of systemic disease in a mouse infection model (Kurupati et al., 2010). However, although dissemination of S. pyogenes to the lungs of infected mice was SpyCEP-dependent, its deletion did not affect persistence in the upper airways (Kurupati et al., 2010). Immunization of mice with SpyCEP significantly reduced dissemination of both S. pyogenes and S. equi in an intramuscular mouse infection model (Turner et al., 2009), suggesting that immunization with CEP could provide cross-protection against a range of streptococci that produce such proteins.

**Resolution of lymph node abscessation and persistence**

The inability of the innate immune response to kill S. equi results in the release of complement-derived chemotactic factors and a massive influx of neutrophils into tonsillar tissues and draining lymph nodes to which S. equi has metastasized. As S. equi numbers increase and abscesses form, so the lymph nodes swell and eventually (approximately 4 to 21 days post-infection) rupture either externally through the horse’s skin or, in the case of the retropharyngeal lymph nodes, internally into the guttural pouch. This air-filled sack is an enlargement of the eustachian tube and drains into the nasal cavity. Drainage of abscess material...
into the nasal cavity from the guttural pouch contributes to the mucopurulent nasal discharges commonly observed during strep. In most horses clearance of infection accompanies drainage. However, in up to 10% of cases complete drainage does not occur. Residual pus becomes inspissated to form chondroids, which may contain live S. equi that remain in the guttural pouch for several years (Newton et al., 1997; Verheyen et al., 2000). Those persistent infections in ‘carrier’ horses are likely to have been critical to the global success of S. equi because intermittent shedding from the guttural pouches of otherwise normal horses contributes to the interepizootic maintenance of the disease. However, with the exception of SeM (Chanter et al., 2000), little is known about the selective pressures exerted on the surface proteins of S. equi as it persists for long periods in the face of local acquired immune responses.

The SeM protein is of special interest because its N terminus may be truncated or mutated over time in isolates recovered from the guttural pouch (Chanter et al., 2000; Kelly et al., 2006) with 87 alleles currently listed on the online seM database [http://pubmlst.org/seq/mstdbnet/seqdbnet.pl?file=sz_seM.xml (accessed 28/03/11)]. Truncation leads to loss of fibrinogen binding, suggesting this function is not important in the guttural pouch. Changes in the N terminus of SeM may alter a conformational epitope of significance in mucosal IgA and T-cell responses, but does not affect the overall susceptibility of the organism to opsonic antibody (Timoney et al., 2010). It is not known if SeM allelic variants of S. equi vary in virulence. It was thought that strains lacking the N terminus of SeM may be avirulent, and therefore be suitable candidates for the development of live attenuated vaccines. However, challenge of Welsh mountain ponies with a dose of 2 × 10⁷ c.f.u. of one such mutant led to the rapid onset of clinical signs (N. Chanter, unpublished data).

Vaccination

Although S. equi can be resolved into 87 different types based on the sequence of the 5’ region of the SeM encoding gene, isolates of S. equi are actually very closely related and can only be differentiated into two sequence types by multilocus sequence typing (Webb et al., 2008). Vaccination of mice with recombinant SeM was protective (Meehan et al., 1998), but these promising results were not repeated on vaccination and challenge of horses (Sheoran et al., 2002). A live attenuated strain of S. equi, Equilis StrepE, containing an aroA gene deletion (Kelly et al., 2006), protected 50% of horses from developing lymph node abscesses (Jacobs et al., 2000) suggesting that vaccines that generate responses against multiple bacterial targets could be effective at preventing S. equi infection. Exploiting the emerging S. equi genome sequence data and a mouse infection model (Flock et al., 2004, 2006) led to the identification of a combination of seven S. equi antigens that proved to be highly protective in Welsh mountain ponies (Guss et al., 2009). These components share similar function or amino acid identity with their S. pyogenes counterparts, and a similar combination of S. pyogenes antigens may prove efficacious in humans.

Conclusions

Long-term occupation of the guttural pouch environment may be a driving force behind several of the observed gene loss events from the S. equi genome. Analysis of strains recovered from this environment may lead to further improvements in the efficacy of emerging multicomponent subunit vaccines and shed light as to how and why S. equi has evolved to become a pathogen restricted to one host.

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