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Vaccination with a live multi-gene deletion strain protects horses against virulent challenge with *Streptococcus equi*

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ABSTRACT

Strangles, caused by *Streptococcus equi* subspecies *equi* (*S. equi*) is one of the most frequently diagnosed infectious diseases of horses and there remains a significant need to develop new preventative vaccines. We generated a live vaccine strain of *S. equi* containing deletions in six genes: *sagA*, *hasA*, *aroB*, *pyrC*, *seM* and *recA*, which was administered to nine Welsh mountain ponies *via* the intramuscular route. Four vaccinated ponies developed adverse reactions following the first vaccination from which the live vaccine strain was isolated. Two of these ponies were withdrawn from the study and seven ponies received a second vaccination, one of which then developed an adverse reaction. Nine control ponies injected with culture media alone developed no adverse reactions. Following challenge with a virulent strain of *S. equi*, none of the seven vaccinated ponies had developed clinical signs of strangles eleven days post-challenge, compared to six of nine control ponies over the same period ($P = 0.0114$). A lymph node abscess was identified in one of the seven vaccinated ponies at post-mortem examination, whilst all nine control ponies had at least one lymph node abscess ($P = 0.0009$). Three of the six vaccinated ponies that were protected from strangles had not developed an adverse reaction following vaccination, suggesting that a better understanding of the pro-inflammatory responses to *S. equi* could lead to the development of a live attenuated vaccine against strangles that is safe for administration *via* intramuscular injection.

1. Introduction

The host-restricted pathogen *Streptococcus equi* subspecies *equi* (*S. equi*) is the causative agent of equine strangles, one of the most frequently diagnosed infectious diseases of horses worldwide, which is characterized by abscessation of the lymph nodes of the head and neck. *S. equi* shares a common phage pool, and several horizontally acquired virulence-associated genes, with the host-restricted human pathogen *Streptococcus pyogenes* [1]. The global population of *S. equi* is closely related, with only six different multilocus sequence types (STs) identified to date [http://pubmlst.org/szooepidemicus/last (24.09.14)] [2]. Despite this apparent lack of diversity, the success of vaccines against strangles has been limited.

The only available strangles vaccine in Europe, Equilis StrepE, is a live attenuated *aroA* deletion mutant, which is administered into the upper lip [3]. In separate experiments, this vaccine protected five of five horses and then two of four horses from developing lymph node abscesses following intra-nasal challenge two weeks post-second intra-lip vaccination ($P = 0.0476$ and $P = 0.4286$, respectively) [3]. Intranasal administration conferred no protection, but intramuscular administration of this vaccine appeared to be efficacious, protecting all three vaccinated animals [3].

Abbreviations: cfu, colony forming units; DIVA, differentiation of infected from vaccinated animals; DMEM, Dulbecco's modified Eagle's medium; iELISA, indirect enzyme-linked immunosorbent assay; mpep1, N-terminal fragment of SeM; OD_{450nm}, optical density at 450 nm; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 0.05% Tween 20; PBSTN, phosphate buffered saline containing 0.05% Tween 20 and 1% non-fat milk; *S. equi*, *Streptococcus equi* subspecies *equi*; *Se*4047, *Streptococcus equi* subspecies *equi* strain 4047; SLS, streptolysin S; ST, sequence type; THA, Todd Hewitt agar; THAE, Todd Hewitt agar containing erythromycin at 0.5 μg ml^{-1}; THB, Todd Hewitt broth; THBE, Todd Hewitt broth containing erythromycin at 0.5 μg ml^{-1}; THBS, Todd Hewitt broth with 10% foetal calf serum; V1, first intramuscular vaccination; V2, second intramuscular vaccination.

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However, injection site reactions, from which the vaccine strain could be recovered, precluded the practical administration of this vaccine *via* the intramuscular route [3–5]. The safe administration of a strangles vaccine *via* the intramuscular route would have significant benefits, being more convenient for veterinarians to use and potentially permitting its administration alongside other equine vaccines such as those that protect against influenza virus and tetanus.

The Equilis StrepE strain produces SeM and SEQ2190 surface proteins that are used in serological assays for the detection of horses that have been exposed to *S. equi* [6]. Therefore, horses vaccinated with Equilis StrepE are likely to test positive using this serological assay, requiring them to be examined further to determine if they present an infectious risk to naïve horses before they can be moved to other premises [7]. A vaccine which permits the differentiation of infected from vaccinated animals (DIVA) would facilitate the movement of vaccinated horses between premises and enable the identification of vaccinated animals that have subsequently been exposed to *S. equi* without developing clinical signs of disease.

The evasion of host immunity is essential for the survival and virulence of pathogenic bacteria. *S. equi* deploys an array of anti-phagocytic factors and toxins that confound the innate immune response. A hyaluronic acid capsule mimics the same molecule produced in vertebrate tissue and shields the bacterium from immune recognition [8]. An anti-phagocytic M-like surface protein, SeM, binds fibrinogen and IgG4 and IgG7 sub-classes [9,10]. Binding fibrinogen masks C3b-binding sites on the bacterial surface reducing the rate of phagocytosis [9–11] and deletion of SeM attenuated *S. equi* in an intra-peritoneal mouse infection model [10]. Production of a haemolytic toxin, streptolysin S (SLS) by *S. equi* is likely to damage host tissue and inhibit neutrophil recruitment analogous to SLS of *S. pyogenes* and group G streptococcus [12–14]. We hypothesized that the intramuscular route could be accessed if the vaccine strain was more susceptible to immune-mediated killing. Here we report the safety and efficacy of a new vaccine strain, based on *S. equi* strain 4047 (*Se*4047), which contained un-marked deletions in six genes: *sagA*, *hasA*, *seM*, *aroB*, *pyrC* and *recA*.

2. Materials and methods

2.1. Bacterial growth and DNA isolation

Deletion strains were derived from *Se*4047, a field isolate responsible for causing strangles in a New Forest pony in Hampshire in 1990 for which a complete genome sequence is available (Accession number NC_012471.1) [1]. Strains were grown overnight at 37 °C on COBA Strep select plates (bioMérieux) in an atmosphere containing 5% CO_2. Genomic DNA was isolated from single colonies using GenElute spin columns (Sigma) according to the manufacturer's instructions.

2.2. Allelic replacement mutagenesis

Internal gene deletions were introduced into *Se*4047 through an allelic replacement strategy using the pG$^+$host9 plasmid [15], which has previously been described for the production of a *ΔprtM* mutant [16]. Briefly, approximately 500 bp fragments of DNA that flank the desired sequence to be deleted were generated by PCR using the primers listed in Table S1, and cloned into the pG$^+$host9 plasmid *via* its *Eco*RI and *Sal*I restriction sites to generate plasmids pGsagA, pGhasA, pGseM, pGaroB, pGpyrC and pGrecA, which contain DNA flanking the desired internal portion of the relevant target coding sequence. In order to generate the vaccine strain, *Se*4047 was transformed with pGsagA and transformants were subjected to two rounds of homologous recombination. The first recombination event, leading to the integration of pGsagA into the *Se*4047 chromosome, was achieved by growing transformants in Todd Hewitt broth (THB) at 28 °C overnight and then increasing the temperature to 37 °C for 3 h. Integrants were selected following growth on Todd Hewitt agar containing erythromycin at 0.5 μg ml^{-1} (THAE) overnight at 37 °C. Integrants were inoculated into Todd Hewitt broth containing erythromycin at 0.5 μg ml^{-1} (THBE) and grown at 37 °C overnight followed by dilution into THB and incubation at 28 °C for a further 48 h. Incubation at the permissive temperature (28 °C) allowed plasmid replication and facilitated the second recombination event. Bacteria were plated on Todd Hewitt agar (THA) and grown at 37 °C to promote the loss of free plasmid. Putative mutant colonies were sub-cultured onto fresh THA and THAE plates to confirm their erythromycin sensitivity. The presence of the *ΔsagA* mutant allele in the chromosome of putative mutants was determined by PCR using the primers listed in Table S1 followed by DNA sequencing on an ABI3100 DNA sequencer with BigDye fluorescent terminators. Additional deletions in *hasA*, *seM*, *aroB*, *pyrC* and *recA* were introduced sequentially following the protocol described above, into the *ΔsagA* mutant of *Se*4047 to yield a vaccine strain lacking fragments of all six genes. Mutant strains of *Se*4047 lacking *hasA*, *seM* or *aroB* were also generated. PCR was performed across each of the deleted target genes using the primers listed in Table S1 and the products sequenced on an ABI3100 automated sequencer to confirm that the desired deletions had been generated.

2.3. Air-interface infection model

Air-interface respiratory tract organ cultures accurately predicted the behaviour of mutant strains lacking *lgt* and *prtM* when these mutants were used to infect a susceptible natural host [16]. This *in vitro* model was used in this study to determine the effects of gene deletions on the ability of *S. equi* to damage and attach to equine tissue. Organ cultures were constructed using equine upper respiratory tract tissues (nasal turbinate, guttural pouch and trachea) as described previously [16]. Tissues were obtained from an abattoir and washed in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (penicillin 100 U ml^{-1}; streptomycin 50 μg ml^{-1}; gentamicin 100 μg ml^{-1}; amphotericin 2.5 μg ml^{-1}) for 4 h to remove commensal flora. Following further washing in DMEM to remove residual antibiotics and amphotericin, tissues were dissected into pieces approximately 5 mm^2 and mounted on agarose platforms surrounded by 2.5 ml DMEM, in six-well cell culture plates. Organ cultures were maintained in a humidified 5% CO_2 incubator at 37 °C. The viability of the air-interface organ cultures was assessed using polystyrene bead clearance. Contamination was monitored by running a bacteriology loop around all four edges of the culture pieces and streaking onto horse blood agar plates. Any tissue pieces in which contamination was detected were discarded. Organ culture pieces were infected with a 10 μl suspension containing 1×10^5 colony forming units (cfu) of wild-type *S. equi* 4047, *S. equi* mutants, or were mock-infected with THB. Colonization of organ culture pieces was quantified by measuring viable counts (six organ culture pieces per time point) of adherent bacteria at 2 h and 24 h post-infection. Organ culture pieces were vortexed for 15 s in phosphate buffered saline (PBS) to remove non-adherent bacteria and then homogenized before plating serial ten-fold dilutions onto THA and enumerating colonies. Data represent the means and standard deviations of six independent experiments using tissues from six different horses. Changes in the surface features of organ culture pieces (two per time point) in response to infection at 24 h post-infection were identified by morphometric analysis of scanning electron microscopy images of the epithelial surface. Tissues

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