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Original Research

Adhesion of *Streptococcus equi* to Air–Liquid Interface Ex Vivo Cultures of the Equine Guttural Pouch Mucosa Is Inhibited by Heparin

Gian Enrico Magi [a,*], Maria Patricia Arias [b], Silvia Vincenzetti Vincenzetti [a], Anna Rita Attili [a], Vincenzo Cuteri [a], Silvia Preziuso [a], Andrew Stephen Waller [c], Giacomo Rossi [a]

[a] *School of Bioscience and Veterinary Medicine, University of Camerino, Camerino, Italy*
[b] *CES University, Medellín, Colombia*
[c] *The Animal Health Trust, Newmarket CB8 7UU, United Kingdom*

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ABSTRACT

The ability of *Streptococcus equi* to persist within the guttural pouches of recovered horses is critical to the transmission and success of this important pathogen. Here, we quantify the adherence of five different strains of *S. equi* to air–liquid interface three-dimensional ex vivo cultures of guttural pouch mucosa and demonstrate that the addition of heparin significantly reduces bacterial adhesion. Our data suggest that the persistence of *S. equi* in the guttural pouch may be enhanced through binding to glycosaminoglycans that are present on the mucosal surface. Further research is warranted toward the identification of the specific receptors involved in this interaction.

1. Introduction

Streptococcus equi subsp. *equi* (*S. equi*) is the causative agent of strangles, which is the most common upper respiratory airway infection of horses worldwide with significant economic impact [1–3]. Affected animals present signs of fever, nasal discharge, and abscesses of the lymph nodes in the head and neck [4]. After the resolution of acute clinical signs of disease, *S. equi* can persist within the guttural pouches of a proportion of recovered horses permitting onward transmission to naïve animals [5]. The ability of *S. equi* to persist in the guttural pouch environment is critical to the success of this important pathogen but requires a different array of properties when compared with those used to cause strangles. This conflict between acute and persistent infection has been investigated at the genome level where mutations in proteins, including the cell wall–anchored surface protein SeM [6], which encodes a fibrinogen-binding protein involved in the ability of *S. equi* to evade phagocytosis [7,8]. SeM is known to be an important virulence determinant, and mutation of SeM during persistent infection was proposed to assist *S. equi* to evade host immune responses, while permitting onward transmission to naïve animals. The variation of the 5'region of the SeM gene is exploited in single locus typing methods that differentiate strains of *S. equi* [9,10]. Interestingly, the differentiation of strains via the SeM gene sequence was concordant with the differentiation of strains based on their core genome sequence, albeit with reduced capacity to resolve closely related strains [6]. The decay of the *S. equi* genome, including the loss of the N-terminal region of SeM, occurs as the organism adapts to life in the guttural pouch [6,11]. The loss of SeM may confer a competitive advantage in the face of a

* Corresponding author at: Gian Enrico Magi, Via Circonvallazione 93/95, 62024 Matelica (MC), Italy.
E-mail address: gianenrico.magi@unicam.it (G.E. Magi).

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convalescent immune response, but no isolates have yet been recovered from acute cases of strangles that contain deletions in the SeM gene [6]. The M protein of *Streptococcus pyogenes*, which shares 30% amino acid identity with SeM of *S. equi*, binds to dermatan sulphate, highly sulphated fractions of heparin sulphate and heparin of human epithelial cells, whereas streptococci deficient in M protein expression failed to interact with glycosaminoglycans (GAGs) [12]. In a study, Parillo et al [13] demonstrated that secretions produced by guttural pouch mucosal cells are particularly rich in chondroitin sulphate, heparin sulphate, and heparin; therefore, these GAGs may represent important binding sites for *S. equi* colonisation. We hypothesized that strains with different SeM alleles would bind differently to explants of the guttural pouch. Here, we report the quantification of the binding of five different strains of *S. equi* to the surface of the guttural pouch and determine the effects of heparin on this important interaction between host and pathogen.

2. Material and Methods

2.1. Bacterial Strains and Growth Conditions

Five strains of *S. equi* known as strain 303 (isolated from Canada in 1999 and possessing SeM allele 28), strain 7344 (isolated from Hampshire, UK in 2003, SeM 9), strain 4047 (isolated from Hampshire, UK in 1990, SeM 3), strain 181063 (isolated from Australia in 1999, SeM 15), and strain 3154 (isolated from Suffolk, UK in 2004, SeM 12) were used in the study. All bacteria were grown on tryptone soya broth (Oxoid, Italy) for 24 hours and then plated onto nutrient agar (Biolife, Italy) supplemented with 0.5% sodium chloride, 0.2% yeast extract (Oxoid, Italy), 10% defibrinated horse blood, and 4% *Streptococcus* Selective Supplement (Oxoid, Italy).

2.2. Tissue Collection

Guttural pouch mucosa samples were obtained from adult horses at the time of slaughter at an abattoir in Italy. The horses used in this study were examined, paying special attention to the respiratory airways and lymph nodes of the head to ensure that they did not have signs of respiratory infection. The age and sex of the horses were not considered in this study. Samples of approximately 10 cm of the guttural pouch wall from the right and left pouches were taken as well as from the medial compartment. The tissue samples were placed into 100-mL capped bottles filled with prewarmed Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Italy) containing 10 mM of glutamine, 80,000,000 U of gentamicin mL^{-1}, 40,000,000 U of streptomycin mL^{-1}, and 400 mg of amphotericin mL^{-1} and incubated for 2 hours at 37°C to remove commensal flora. They were then transferred to another 100-mL bottle with prewarmed DMEM without antibiotics and incubated for another 2 hours at 37°C. During this period, the medium was changed twice to remove residual antibiotics from the tissues.

2.3. Air–Liquid Interface Organ Cultures Construction and Infection

Air–liquid interface organ cultures were constructed using the equine guttural pouch mucosa explants, based on methods described for human and canine tissues [14,15]. After the washes, the tissue samples were placed on a sterile Petri dish, to carefully remove the connective tissue that adhered to the mucosa. Next, tissue samples approximately 6 mm wide were made with a 6-mm biopsy punch (KAI Industries, Japan). Biopsies were mounted, mucosa side uppermost, at an air–liquid interface directly on agarose plugs. Agarose plugs were made by pipetting 1 mL of 1% agarose in sterile distilled water, into a well of a 24-well cell culture plates. After the agarose had set, the plugs were transferred into 6-well cell culture dishes. Organ culture biopsies were infected with the five strains at a concentration of 2×10^5 colony-forming units (CFU) of bacteria, or no bacteria controls, in DMEM for 4 hours and 24 hours at 37°C in an atmosphere containing 5% CO_2. Where appropriate, DMEM was supplemented with 0.25 mU/mL of heparin (Teva, Italy). For each experiment, ciliary motility was evaluated from one biopsy as described by Santamaria et al [16], to evaluate ciliary motion. If immotile cilia were observed, the tissue collected was discarded. For each experiment, eight culture samples were used for microbiological analysis (three cultures infected, three cultures infected and supplemented with heparin, two cultures as control) and three samples was used for morphometric analysis (one culture infected, one cultures infected and supplemented with heparin, one culturea as control). After incubation, organ culture samples were washed for 10 seconds in phosphate buffer saline solution to remove nonadherent bacteria and homogenized with ultra turrax equipment (IKA-Lab, Italy). Tenfold serial dilutions of the resulting suspension were plated onto Strep agar culture plates and incubated in a 5% CO_2 containing atmosphere for 24 hours at 37°C for enumeration of adherent bacteria. Experiments were performed in triplicate.

Table 1
Bacterial count of five strains of *Streptococcus equi*.

Incubation Time Heparin Treatment	Strain 303	Strain 3154	Strain 181064	Strain 7344	Strain 4047
4 hr	$2 \times 10^6 \pm 0.8$	$1.6 \times 10^4 \pm 0.9$	$2.6 \times 10^6 \pm 0.9$	$3.6 \times 10^6 \pm 1.2$	$2.6 \times 10^5 \pm 0.4$
24 hr	$2.3 \times 10^6 \pm 0.4$	$1.6 \times 10^4 \pm 0.4$	$2.6 \times 10^6 \pm 0.4$	$1.6 \times 10^6 \pm 0.4$	$3 \times 10^5 \pm 0.8$
Heparin treatment (4 hr)	$2.3 \times 10^4 \pm 1.2$	$1.3 \times 10^3 \pm 0.4$	$3.6 \times 10^4 \pm 0.9$	$2 \times 10^4 \pm 0.8$	$3 \times 10^3 \pm 1.4$
Heparin treatment (24 hr)	$2.3 \times 10^4 \pm 0.4$	$3 \times 10^3 \pm 0.8$	$3.6 \times 10^4 \pm 1.2$	$2.3 \times 10^4 \pm 0.4$	$2.3 \times 10^3 \pm 0.9$

Abbreviations: CFU, colony-forming unit; SD, standard deviation.
CFU mean values ± SD are shown. Three cultures were analyzed at each time interval.