Controlling Wound Bacteria and Biofilm

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1. Introduction

Recognition of the role that biofilms play in the persistence of chronic wounds and the lack of response to treatment in horses is increasing. Preventing biofilm development includes the following three main strategies: effective wound cleansing and debridement to reduce bacterial counts in wounds, appropriate use of advanced dressings, and use of topical antimicrobial agents. Once biofilms are formed, eradicating them involves striking a balance between improving the wound environment without harming the native cells that are integral to the healing process, primarily through repeated lavage and debridement combined with topical antimicrobial therapy. The key points in this review are to understand why and how biofilms form, to recognize clinical indicators that biofilms have formed in equine wounds, and to be familiar with current diagnostic options and treatment strategies to eradicate biofilms. Clinical scenarios for cases in which biofilms developed and were treated will be discussed.

2. Understanding Biofilms

Bacterial biofilms are organized communities of bacteria that are typically attached to a surface (sessile) and enveloped in a 3-dimensional extracellular matrix (also known as extracellular polymeric substance [EPS]) that includes water, proteins, polysaccharides, glycolipids, bacterial DNA, and potentially other microbes that are benefiting from the protected environment.1-3 Bacteria that produce biofilms are capable of surviving and growing at a slower rate in local environments depleted of nutrients and oxygen, termed phenotypic heterogeneity.4 Biofilm formation is divided into the following three main stages: bacterial attachment, growth, and detachment.5 Planktonic (free-floating) bacteria adhere to a surface within minutes (stage 1), and the individual bacterium alter their phenotype and secrete extracellular matrix after 6 to 12 hours of attachment, growing and maturing based on cell-to-cell signaling called “quorum-sensing” (stage 2).6 Biofilms reach maturity within 2 to 4 days, and then begin to shed free-floating planktonic cells (stage 3), which disperse and attach to other areas of the wound bed. This distribution of cells activates the immune response of the host animal, stimulating the production of exudates that provides further nutrients and promotes survival of the biofilm.7-9 Once biofilms form, bacteria differentiate into a complex community with increased resistance to antibiotics, biocides, and environmental challenges such as desiccation and cells of the innate immune system.10,11 These obstacles in killing bacteria in biofilms may only be overcome if antimicrobials to which the bacteria are sensitive can be delivered at adequate concentrations for a sufficient time.9

NOTES
3. Problems Caused by Biofilms

Polymeric biofilms have been reported in multiple types of equine wounds (acute and chronic, traumatic, and surgical). Factors that may predispose patients to biofilm formation in wounds include the immunocompetence of the patient (age, malnutrition, sepsis, corticosteroid administration, antibody deficiency, chronic stress, or diseases affecting the pituitary-adrenal axis such as pituitary pars intermedia dysfunction [PPID] or Cushing’s disease), inappropriate antibiotic sensitivity, reduced vascular perfusion to region, extensive wound contamination, or the presence of foreign bodies, surgical implants, or sequestra. Preventing biofilm development in acute wounds includes successful wound cleansing and debridement to reduce bacterial counts and the appropriate use of advanced dressings and topical antimicrobial agents. Development of infection involving biofilms has important implications for the management of chronic wounds in horses, as they present unique challenges in diagnosis and are more resistant to typical treatment methods. Wounds with biofilms may not exhibit signs typically associated with infection, although biofilms still prolong and impair healing as bacteria compete for metabolic resources and suppress the host inflammatory response in healing. The presence of biofilms has been shown to delay epithelialization and induce a chronic non-healing inflammatory phase in wounds. Furthermore, bacteria within biofilms are more tolerant to antimicrobial therapy administered topically (antiseptics) or systemically (antibiotics), as well as to the animal's innate immune phagocytic response and environmental stresses as they are protected by their extracellular polymeric matrix. For example, *Staphylococcus aureus* has been demonstrated to be up to 100-fold more resistant to antimicrobial therapy when growing in biofilms versus in planktonic status. Mature biofilms are impervious to commonly used antiseptics, such as hydrogen peroxide, alcohols, bleach, acids, and generators of oxygen radicals, unless these products are used at concentrations toxic to the patient’s cells, which is not recommended. In addition, the ability of the animal's immune response to effectively control microbes decreases as the biofilm matures. Consequently, infections involving biofilms frequently recur following discontinuation of antibiotics. Early recognition of the presence of biofilms in non-healing wounds and targeted treatment are key to the successful management of biofilms in equine practice.

4. Diagnosing Biofilms (Clinical Indications and Laboratory Testing)

Recent studies have demonstrated that biofilms associated with wounds are most commonly polymicrobial bacterial communities that include more bacterial species than are identified by routine culture and sensitivity methods. Traditional culturing techniques are frequently inadequate to comprehensively identify bacterial species associated with biofilms. Biofilms in wounds can only be definitively diagnosed using scanning electron or confocal microscopy techniques or molecular techniques to identify bacterial components within a biofilm, which are not readily available to clinicians. Biofilms have been identified in 60% of chronic wounds and 6% of acute wounds in one study, although more recent work suggests that biofilms are involved in most, if not all, chronic wounds. Multiple studies have documented evidence of biofilms in chronic wounds of horses specifically.

To review, typical methods to assess bacterial burden in wounds may be done qualitatively or quantitatively. Qualitative assessment involves determining types of bacteria in wounds, coupled with sensitivity testing to guide antibiotic choices in treatment. Quantitative bacteriology is rarely performed in veterinary medicine but should be considered when a wound is not progressing as anticipated or the skin graft fails. Bacterial counts greater than 10^5 per gram tissue or mL exudate has historically been considered to indicate active infection, although the number of bacteria needed to produce infection may be dramatically reduced if bacterial virulence is high, foreign material (suture, necrotic debris, foreign body, and implant) are present, or host resistance is decreased. For example, as few as 100 bacteria per gram of tissue or mL exudate may be needed to incite infection with multidrug-resistant isolates and polymicrobial infections with 2 or more different microorganisms act synergistically to result in greater virulence than infection caused by either species alone. Although a single species may make up a biofilm, recent studies have provided evidence that bacterial isolates from chronic equine wounds are frequently polymicrobial, with an average number of species identified of 3.02 ± 1.65 (range, 0–8), and with the bacterial genera involved being similar to those identified in human infections. One study identified *Pseudomonas, Enterococcus*, and *Staphylococcus* members as the most common organisms in chronic wound biofilms in humans. This finding further highlights the inadequacies of traditional culture methods, as molecular analyses of chronic wound samples has revealed far more diverse polymicrobial communities (up to 17 genera per wound), including anaerobic species that are not identified by traditional culture.

Currently, the best diagnostic method available to equine practitioners when biofilms are suspected is to submit a deep tissue biopsy, a swab of the deepest tissues available, or both. Following debridement of the wound, samples should be collected from multiple sites if possible, particularly from the deepest regions of the wound (e.g., pockets and fissures). When practitioners swab wounds for sample collection, the swab should be drawn across the surface of the wound with sufficient pressure to collect a sample of the biofilm itself. Drawing blood in the process of collecting the sample should be avoided, as blood itself contains...
Table 1. Indirect Clinical Indications of Wound Biofilm

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<tr>
<th>Clinical Observation</th>
<th>Biofilm Explanations for Clinical Observation</th>
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<tr>
<td>Excessive moisture associated with wound</td>
<td>Bacteria in biofilms secrete extracellular matrix, and biofilm presence promotes inflammation, resulting in increased exudate.</td>
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<tr>
<td>Autograft or allograft fails on wounds</td>
<td>Applying tissue grafts over biofilms provides a second growth surface and food source, leading to devitalization of graft tissue and increased exudate and inflammation.</td>
</tr>
<tr>
<td>Poor quality granulation tissue (e.g., hypergranular, friable)</td>
<td>Biofilm presence contributes to delayed epithelialization and is frequently associated with poor quality granulation tissue.</td>
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<tr>
<td>Indications of local infection (swelling, sensitivity, redness, heat)</td>
<td>Biofilms promote inflammation and may be a precursor to other clinical indicators of infection.</td>
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<tr>
<td>History of persistent or recurrent infection despite antimicrobial therapy</td>
<td>Biofilm bacterial phenotypes adapt rapidly and may only demonstrate a 1 to 2 log reduction with antibiotic therapy at ( 50 \times \text{MIC} ). Biofilms contain persistor cells that remain once antibiotic therapy is discontinued, seeding and contributing to subsequent biofilm reformation.</td>
</tr>
<tr>
<td>Negative culture results despite clinical suspicion of infection or signs of bacterial colonization</td>
<td>Biofilms metabolize more slowly and are phenotypically different than planktonic bacteria. Standard microbiological culture techniques are not capable of identifying all species present, making bacteria in biofilms difficult or impossible to identify by culture.</td>
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<tr>
<td>Wound remains in chronic inflammatory state and recalcitrant to therapy despite addressing comorbidities</td>
<td>Biofilms are resistant to host inflammatory responses and actually feed off exudate produced by inflammation, further promoting inflammation.</td>
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5. Biofilm Treatment Strategies

Biofilm-based wound-care (BBWC) strategies have been described in several recent consensus documents in human wound care with the goal of providing practical guidelines for clinical management for cases in which biofilms are suspected (Table 2). These strategies emphasize repeated debridement to physically disrupt the biofilm matrix and remove devitalized tissues that serve as nutrients to the microbes. Debridement disturbs the biofilm structure, allowing increased susceptibility to antimicrobial therapies for a period of time that may prevent bacterial reattachment, as immature biofilms are more vulnerable to antibiotics. Two principles of debridement in human wound care described by Wolcott et al. include debriding with the goal of altering the anatomy of the wound by undermining and removing all tissue surfaces that touch one another and removing devitalized and discolored tissue until normal bleeding tissue was encountered. Topical treatments are recommended to be applied within 4 hours of debridement or less, before biofilm reformation. An example of the successful treatment of biofilms (and how quickly they reform in the absence of consistent therapy) includes removal of plaque from dental enamel by using a combination of regular tooth brushing (i.e., debridement) in combination with topical antiseptic mouthwashes. Furthermore, antiseptic mouthwashes are of minimal benefit without preceding flossing and brushing. With this in mind, treatment of biofilms in wounds is recommended in three stages, as follows: physically debriding the biofilm, delaying or preventing reformation of biofilms, and repeating until full resolution of the biofilm is achieved. Debridement of biofilms may be performed sharply (or bluntly with a scalpel blade), using pulsed water-jet irrigation or low-frequency ultrasonic debridement, or mechanically with gauze.
swabbed across the surface of the wound. To minimize discomfort for the patient during this step, it is recommended that horses be sedated and the wound desensitized with local or regional anesthesia. In some instances, general anesthesia may be necessary for the first debridement, if dictated by the patient’s temperament, or if the wound is extensive or not easily accessed. Face protection or surgical masks may be recommended for the veterinarian to protect against multidrug resistant infectious organisms or if using pulsed water-jet irrigation, which may aerosolize organisms to a greater extent. The overall objective of debridement is to remove as much of the biofilm and associated extracellular matrix and devitalized tissue as possible to expose remaining bacteria to antimicrobial agents. Reducing or preventing reconstitution of biofilms following debridement may be achieved in several ways. Surfactants (polyhexamethylene biguanide [PHMB] or polyhexanide) can be used as adjunctive therapies at this time, as they reduce biofilm surface tension, facilitating degradation and removal. Antiseptic agents do not penetrate necrotic debris and are unlikely to reduce bacterial populations deep in the wound bed or without debridement. In general, they should be reserved for use on normal skin and not the wound bed. Examples include acetic acid, alcohols, aluminum salts, boric acid, chlorhexidine, formaldehyde, gentian violet, hexachlorophene, hydrogen peroxide, hypochlorite, iodine, povidone-iodine, merthiolate, permanganate, and silver nitrate. Unlike antiseptics, topical antimicrobial agents provide efficacy against bacteria within the wound bed and have minimal side effects on wound healing (depending on the vehicle). Ideally, antimicrobials are selected based on results of culture and sensitivity, and due to concerns with increasing incidence of resistance, ones used topically should ideally be different to those administered systemically. Topical dressings including silver sulfadiazine (1%) or silver-impregnated wound dressings are currently used to combat biofilms in humans and may be used if antibiotic sensitivities are not available (leaper). However, their efficacy is limited to the first 24 hours, so these dressings must be changed frequently to maintain their effect. If culture and sensitivity results are available, appropriate antibiotics may be applied topically with an enhanced effect. Additionally, topical application of plasma (natural or hyperimmune for the specific pathogen targeted) may be of benefit, as plasma inhibits the adhesion and growth of bacteria. The efficacy of antibiotics delivered regionally (e.g., regional limb perfusion) is also greater immediately after degradation of the biofilm, so timing of perfusion following surgical debridement may improve outcomes. In situations in which both instability and infection are present, implants may be removed or replaced, sonicated, cleaned, autoclaved, and reimplanted using new screws and holes if financially feasible. Alternatively, implants may be removed and unconsolidated fractures managed with a transfraction pin cast or external fixator. The fracture site, bone, and surrounding tissues may be debrided during the sterilization process. The previous screw holes and surrounding region may be packed with absorbable antibiotic eluting materials. Use of surgical drains or leaving the distal aspect of the incision open may facilitate drainage in these cases. Debridement and efforts to impede reconstitution of wound biofilms should be repeated as frequently (daily or at least every other day) and for as long as needed until the infection is resolved. Mature biofilms reform as quickly as 24 hours (up to 72 hours) following debridement, allowing a window of opportunity in which bactericidal drugs may have a greater effect. It is recommended to monitor horses frequently and continue antibiotic therapy until signs of infection have resolved and wound healing is progressing as expected. If improvement is not seen within 3 to 4 days after the initiation of therapy, it is recommended to review all aspects of the case, including repeated physical examination, bloodwork, evaluation of...
suitability of antibiotics administered, and further exploration and debridement of the wound. Repeated bacterial culture and sensitivity may be indicated if signs of infection recur, response to therapy is less than anticipated, or during periods of prolonged antibiotic therapy, following discontinuation of antibiotics, if the infection included multidrug-resistant or polymicrobial agents. In communicating with clients about the cost of care, it is important to emphasize that the greater expense of aggressive debridement in the early stages of wound management typically reduces the duration of therapy and overall costs of treatment long-term. Limitations of current laboratory testing and definitive clinical signs indicative of biofilms make it impossible to determine categorically whether a wound is biofilm free. Therefore, until a stall-side test is available to identify biofilm presence, the clinical progression of wound healing with reduced slough and exudate are the most effective methods of determining response to therapy and resolution of biofilms in tissues.

6. Future Diagnostic Techniques and Treatment Strategies for Biofilms

Future diagnostic tests may indicate more definitively the presence of biofilms and where the biofilm is located within the wound. Stall-side or bedside tests to quantify wound bed protease activity may be one method to indirectly quantify the amount and viability of residual biofilm, as protease activity generally correlates to the amount of active biofilm. Novel techniques to reduce infection associated with biofilms are currently being investigated and developed further. They include quorum-sensing inhibitors (RNAIII-inhibiting peptide [RIP]), surfactants, hydrophobic polycationic coatings, sol gel coatings, covalent antimicrobial tethering, lactoferrin, xylitol, dispersin B, gallium, acetylsalicylic acid, bacteriophages (antibacterial viruses), ultraviolet light, low-voltage pulsed electric fields, and cellular therapeutic options including platelet rich plasma, and mesenchymal stromal cells. Further investigation of methods for improving outcomes in the management of biofilms in randomized controlled clinical trials is indicated.

7. Conclusions

Recognition that most chronic wounds likely involve bacterial biofilms is key to successful treatment. Clinical indications that biofilms may be present include exudate, poor quality granulation tissue, other indications of local infection, negative bacterial culture results despite clinical suspicion of infection, and wounds remaining in a chronic inflammatory state and recalcitrant to therapy. The mainstays of therapy to reduce biofilm bacterial burden in wounds are repeated debridement and timely use of targeted topical antimicrobials and surfactants that have minimal cytotoxicity to native tissues. Improved diagnostic tools in the future to detect the presence of biofilms and monitor response to treatment in a more sensitive manner as well as adjunctive therapies to degrade biofilms may improve outcomes.

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Declaration of Ethics

The Authors have adhered to the Principles of Veterinary Medical Ethics of the AVMA.

Conflict of Interest

The Authors have no conflicts of interest.

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