Review of the role of biofilms in chronic wounds in horses: clinical indications and treatment strategies

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July 11, 2023

Abstract

Recognition of the role that biofilms play in the persistence of chronic wounds and lack of response to therapy in horses is increasing. Prevention of biofilm development in early stages of wound care involves three primary strategies: wound debridement and cleansing to reduce bacterial counts in the wound bed, appropriate use of advanced wound dressings, and implementation of topical antimicrobial agents. Once formed, eradication of biofilms requires elimination to improve the wound environment for contraction and epithelialization while not further harming the native cells integral to the healing process, which is achieved predominantly through repeated lavage and debridement combined with topical antimicrobial therapy. This review will establish why and how biofilms form, how to recognize clinical indications that biofilms have formed in equine wounds, and to review current diagnostic options and biofilm-based wound care (BBWC) strategies to eradicate biofilms. Clinical scenarios for cases in which biofilms developed and were successfully treated will be presented. This review will advance practitioners' understanding of the presence and role of biofilms in chronic wounds and provide an updated summary of recommended treatment strategies.

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Summary

Recognition of the role that biofilms play in the persistence of chronic wounds and lack of response to therapy in horses is increasing. Prevention of biofilm development in early stages of wound care involves three primary strategies: wound debridement and cleansing to reduce bacterial counts in the wound bed, appropriate use of advanced wound dressings, and implementation of topical antimicrobial agents. Once formed, eradication of biofilms requires elimination to improve the wound environment for contraction and epithelialization while not further harming the native cells integral to the healing process, which is achieved predominantly through repeated lavage and debridement combined with topical antimicrobial therapy. This review will establish why and how biofilms form, how to recognize clinical indications that biofilms have formed in equine wounds, and to review current diagnostic options and biofilm-based wound care (BBWC) strategies to eradicate biofilms. Clinical scenarios for cases in which biofilms developed and were successfully treated will be presented. This review will advance practitioners' understanding of the presence and role of biofilms in chronic wounds and provide an updated summary of recommended treatment strategies.

Introduction

Bacterial biofilms are organized communities of bacteria attached to a surface and enveloped in a three-dimensional extracellular matrix. A recent systematic review and meta-analysis of wound care literature in humans reported the prevalence of biofilms in chronic wounds to be 78.2% (Malone *et al.,* 2017). Consensus guidelines for the identification and treatment of biofilms have further stated that biofilms should be assumed to be involved in most, if not all, chronic non-healing wounds (Schulz *et al.,* 2017). Furthermore, multiple studies to date have documented evidence of biofilms in chronic wounds of horses specifically (Freeman *et al.,* 2009; Westgate *et al.,* 2011). The high prevalence of biofilms in non-healing wounds, the frequency with which equine practitioners treat wounds in daily practice, and the increasing reported incidence of multi-drug resistant bacterial strains in equine practice in general (Herdan *et al.,*2012; Loncarac *et al.,* 2014; Theelin *et al.,* 2014; van den Eede *et al.,* 2012), highlight the need for implementation of more advanced training in wound care strategies to address these clinical scenarios.

The purpose of this review is to summarize the current literature describing problems caused by bacterial biofilms in wounds, clinical indications that biofilms are involved, laboratory testing to improve biofilm detection, and biofilm-based wound-care (BBWC) strategies to provide clinicians with practical guidelines for case management where biofilms are suspected. Recommendations for antimicrobial duration in veterinary practice further support administration for the shortest effective duration to reduce risk of development of resistant pathogens (Hansen *et al.,* 2014; Gandini *et al.,* 2022); therefore, local surgical and topical techniques to address biofilm formation will be emphasized to minimize unnecessary systemic antimicrobial administration. Early recognition of the presence of biofilms in non-healing wounds and targeted treatments are key to the successful management of biofilms in equine practice (Pezzanite *et al.,*2021).

Understanding the role of biofilms in wounds

Biofilm formation is divided into three main stages: bacterial attachment, growth, and detachment (Lappin-Scott and Bass 2001). In stage one, planktonic (free-floating) bacteria adhere to surfaces within several minutes (Parsek *et al.,* 2005). In stage two, individual attached bacteria (*i.e.,* 'sessile') secrete a three-dimensional extracellular matrix (also known as extracellular polymeric substance (EPS)) that includes water, proteins, glycolipids, polysaccharides, bacterial DNA, and potentially other microbes benefiting from the protected environment which makes up 90% of the biomass of the biofilm itself (Clutterbuck *et al.,* 2007; Jacques *et al.,* 2010; Wolcott *et al.,* 2008a; Percival, McCarty, Lipsky 2015; Flemming and Wingender 2010). This occurs within 6 to 12 hours of attachment and the biofilm continues to grow based on coordinated cell-to-cell signaling known as 'quorum-sensing.' (Parsek *et al.,* 2005; Prada & Săndulescu, 2019; McCarty *et al.,* 2012). In stage three, biofilms reach maturity within 2 to 4 days and shed free-floating planktonic cells which disperse and attach to other areas of the wound bed (Kostakioti, Hadjifrangiskou, & Hultgren 2013). This cell distribution activates the host immune response, which further stimulates production of exudates that provide nutrients and promote survival of the biofilm (Orsini *et al.,* 2017; Dart *et al.,*2017a; Stewart and Richardson, 2019), and may lead to additional complications for the host animal, including bacteremia or bacterial colonization of distant anatomical sites (Bjarnsholt *et. al.,*2013).

Predisposing factors to biofilm formation include the presence of foreign bodies, sequestra and surgical implants, reduced vascular perfusion to the anatomical region, inappropriate antimicrobial sensitivity, and the immune status of the patient (age, sepsis, malnutrition, antibody deficiency, chronic stress, corticosteroid administration, or underlying diseases including pituitary pars intermedia dysfunction [PPID] or Cushing's disease) (Seth *et al.,* 2012, Orsini *et al.,* 2017). Strategies to prevent biofilm development in acute wounds include wound debridement and cleansing to reduce bacterial counts and appropriate use of advanced dressings and topical antimicrobial agents. Addressing systemic conditions (*e.g.,* Cushing's disease in horses) may promote more rapid bacterial clearance and healing in immune-incompetent patients as well. Furthermore, the ability of the host's immune response to effectively control microbes decreases as the biofilm matures. As a consequence, infections involving biofilms frequently recur following discontinuation of antimicrobials (Dart *et al.,* 2017b), emphasizing that early recognition of treatment of both the wound and the animal's systemic health status are key to successful management.

Wounds with biofilms may not necessarily exhibit signs typically associated with infection besides prolonged and impaired healing (Dart*et al.,* 2017a). The presence of biofilms has been demonstrated to delay epithelialization and induce a chronic non-healing inflammatory state (Wolcott *et al.,* 2008; Schierle *et al.,* 2009). However, it is important to note that polymicrobial biofilms, which are considered more pathogenic than monobacterial colonies, have been reported in multiple types of equine wounds, not limited to those considered chronic (*e.g.,* acute or chronic, surgical or traumatic in origin) (Westgate *et al.,* 2001; Freeman *et al.,* 2009; Pastar *et al.,* 2013). Metabolically active, nondividing persister cells, which are tolerant to antimicrobials, are integral to reestablishing biofilms following topical treatments (Kostakioti, Hadifrangiskou and Hultgen, 2003). Specific bacterial species may integrate chromosomal β-lactamase, efflux pumps, and mutations in target antibiotic molecules to evade host defenses. Finally, extracellular DNA (eDNA) present in bacterial biofilms promotes acid-base interactions between bacterial cells and surfaces, therefore playing an essential structural role in both establishing biofilms and protecting cells within the biofilm from environmental challenges (Lewenza *et al.,*2013; Thomann *et. al.,* 2016).

Locally, polymicrobial infections delay wound closure through alteration of cytokine levels and receptors (Pastar *et al.,* 2013). For example, *S. aureus* and *P. aeruginosa* are known to downregulate keratinocyte growth factor 1 expression of fibroblasts, resulting in delayed re-epithelialization through reduction of keratinocyte migration and proliferation (Pastar *et. al.,* 2013). Bacteria in biofilms secrete enzymes (*e.g.,* proteases, elastase, phospholipase) to degrade local host tissues to provide nutrients and to protect bacteria within the biofilm from host immune cells (Michalkiewicz *et al.,* 1999; Flemming and Wingender, 2010; McCarty *et al.,* 2017). For example, the proteases secreted by*Pseudomonas aeruginosa* degrades and inactivates interferon gamma which suppresses innate immune recruitment and reduces elimination of biofilm bacteria (Michalkiewicz *et al.,* 1999). Continuous production of exudate is detrimental to wound healing as the inflammatory process continuously breaks down the ECM (McCarty *et. al.,* 2012) and may degrade growth factors associated with normal wound healing processes (Percival *et al.,* 2015). Various cell types including keratinocytes, fibroblasts, endothelial cells, and inflammatory cells (*e.g.,* monocytes, lymphocytes, and macrophages) express matrix metalloproteinases (MMPs) involved in epithelial repair, wound contraction, and degradation of damaged ECM within the skin (Caley *et al.,* 2015) which is upregulated in wound edge keratinocytes to allow epidermal cell migration across wound beds (McCarty *et al.* 2012). However, in wounds associated with biofilms, the presence of devitalized tissue and abnormal immune cell activity results in excessive production of MMPs which perpetuates ECM destruction propogating the inflammatory response and wound chronicity (Caley *et al.,* 2015; Parnham and Bousfield 2018; Kandhwal*et al.,* 2022). Approaches to restore normal wound healing involve techniques directed towards inhibition of these biofilm virulence factors through effective, sustained debridement of devitalized tissues (Schierle *et al.* , 2009; Parnharm and Bousfield 2018).

Development of infection involving biofilms has important implications in management of wounds in horses, as they present unique challenges in diagnosis and are more resistant to typical treatment methods (Dart*et al.,* 2017a). Bacteria that produce biofilms are able to survive and grow at slower metabolic rates in environments depleted of nutrients and oxygen, termed phenotypic heterogeneity (Donlan *et al.,* 2001; Clutterbuck *et al.,* 2007). Mature biofilms secrete protective enzymes, shielding themselves from host defenses and exterior physiologic changes that may be detrimental to bacterial health (Percival *et al.,* 2015). Once formed, bacteria in biofilms differentiate into complex communities with enhanced resistance to environmental challenges (*e.g.,* cells of the innate immune system, desiccation, etc.), biocides, and antibiotics (Costerton*et al.,* 1999; Fux *et al.,* 2005) and variable morphology depending on nutrient availability (Klausen *et al.,* 2003, Flemming and Wingender, 2010). As a result, bacteria within biofilms are more tolerant to the host immune response, antimicrobial therapy administered systemically (antibiotics) or topically (antiseptics) including hydrogen peroxide, alcohols, bleach, oxygen radical generators and acids (unless administered at concentrations toxic to the animal's cells) (Clutterbuck *et al.,* 2007). For example,*Staphylococcus aureus* has been shown to be up to 100 times more resistant to antimicrobials when in biofilm versus planktonic form (Leid*et al.,* 2002). These challenges in addressing 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 to achieve bactericidal

activity (Stewart and Richardson, 2019).

Diagnosing biofilms – laboratory testing and clinical indications

Traditional bacterial culturing techniques are generally considered inadequate to comprehensively identify bacterial species associated with biofilms (Kirketerp-Moller *et al.,* 2008). Diagnosis of biofilms in wounds can only be definitively made using scanning electron or confocal microscopy imaging or molecular techniques to identify bacterial components, which are not readily available modalities to clinicians (Wolcott and Rhoads 2008; Percival *et al.,* 2015; Dart*et al.,* 2017; Schulz *et al.,* 2017; Hurlow *et al.,*2015). Recent studies have demonstrated that biofilms associated with wounds are most commonly polymicrobial communities, with an average number of 3.02 +/- 1.65 species identified (range, 0-8) (Westgate*et al.,* 2001; Freeman et al. 2009). Genera identified were similar to those found in human infections, with *Pseudomonas, Enterococcus* , and *Staphylococcus* species being most common (Wolcott and Rhoads 2008; James *et al.,* 2008; Dowd *et al.,*2008; Darvishi *et. al.,* 2021). However, molecular analyses of chronic wound samples have revealed far more diverse polymicrobial communities with up to 17 genera per wound, including anaerobic species not identified by routine culturing, and further highlighting the challenges faced by clinicians in accurately identifying and treating bacterial species contained within biofilms (James *et al.,* 2008; Han *et al.,* 2011).

Standard methods to assess bacterial burden in wounds include qualitative and quantitative techniques (Hendrickson 2019a). Qualitative assessment determines the genera of bacteria found in wounds and is coupled with sensitivity testing to provide clinicians basis for antibiotic choices in treatment. Quantitative bacteriology methods are less commonly performed in veterinary medicine but should be considered in cases when wound healing is not progressing as anticipated or following skin graft failure. Active infection has typically been considered to be the case in situations where bacterial counts are found to be greater than 10^5 per gram tissue or mL exudate (Robson and Heggers, 1969). However, the number of bacteria required to establish an infection is reduced in situations where the patient's bacterial resistance or immunocompetence is decreased, foreign material is present including implants, sutures, foreign bodies or necrotic debris, or bacterial virulence is high (Bowler 2003). In polymicrobial infections, as is most typical of those involving biofilms, multiple microorganisms act synergistically to result in greater virulence compared to an infection caused by either species alone (Serra *et al.,* 2015). In cases involving multidrug resistant isolates, as few as 100 bacteria per gram tissue or mL exudate may be sufficient to incite infection (Rodeheaver *et al.,* 1974).

The best diagnostic method currently available to clinicians in equine practice when biofilms are suspected is submission of a deep tissue biopsy or swab of the deepest tissues available (or both) for bacterial culture and sensitivity to guide future treatment practices (Dart*et al.,* 2017). In general, tissue samples, while being more invasive to collect, are more likely to yield reliable culture results compared to swabs (Westgate *et al.,* 2001; Freeman *et al.,*2009). Ideally, submission of tissue samples should be performed prior to beginning or altering antimicrobial protocols; however, if considered necessary to collect samples while horses are currently receiving antimicrobial, it is recommended to notify the receiving laboratory of the horse's current regimen and when the most recent dose was received in relation to sample collection (Orsini *et al.,* 2017). Following superficial wound debridement, tissue samples should be collected from within the deepest regions of the wound (*e.g.,* fissures or pockets in the wound bed) and from multiple sites if possible to avoid false positive results (Sen *et al.,* 2012; Rhoads *et al.,*2012). If tissue swabs are collected, the swab should be drawn across the wound surface with sufficient pressure to collect the biofilm itself while avoiding drawing blood which contains antimicrobial elements that may affect culture results. Positive culture results should be interpreted with the assumption that the full microbial spectrum is likely underrepresented with currently available techniques.

In lieu of obtaining a positive culture result or if submission is not an option due to financial or other case-related considerations, diagnosis of biofilms in wounds may be based on clinical indications (**Table 1**). Clinical findings consistent with biofilm presence include indicators of inflammation (heat, swelling, pain, redness), persistent or recurrent infection despite administration of antimicrobial therapy or recurrence following antibiotic discontinuation, excessive wound moisture/exudate, poor quality granulation tissue, history of negative culture findings despite clinical suspicion of infection, or in general a wound that remains

in a chronic and recalcitrant inflammatory state despite standard treatment and evaluation of the patient for comorbidities (*e.g.,*immunosuppression). In summary, culture findings to diagnose biofilms are unreliable and observation of clinical indications that biofilms are present in the wound bed should prompt practitioners to implement wound care strategies directed specifically at addressing and reducing biofilm formation in wounds.

Biofilm-based wound-care treatment strategies

4.1. Biofilm-based wound-care guidelines - Recent consensus documents in human wound care have described biofilm-based wound-care (BBWC) strategies to provide practical guidelines for case management in which biofilms are suspected (Wolcott and Rhoads, 2008b; Schultz *et al.,* 2017; Metcalf *et al.,* 2014; Bianchi*et al.,* 2016) (**Table 2**). Biofilm treatment is recommended in three stages: 1) physical debridement of the biofilm, 2) topical treatment to delay or prevent reformation, and 3) repeated therapy until full resolution is achieved (Orsini *et al.,* 2017). These strategies emphasize that repeated debridement to physically disturb the biofilm structure is necessary to disrupt the matrix and remove devitalized tissues that serve as nutrients to the microbes involved and allow increased susceptibility to antimicrobial therapies for a period of time to prevent bacterial reattachment as immature biofilms are more susceptible to antimicrobials (Dart *et al.,*2017). Implementation of a multimodal therapeutic strategy to address biofilms has a reportedly higher success rate compared to antimicrobials alone (Wu *et al.,* 2015).

4.2. Debridement principles - The overall objective of debridement is to remove as much devitalized tissue, biofilm and associated extracellular matrix as possible to expose the remaining bacteria to antimicrobial agents. The organization and complex physiology of mature biofilms increases their resistance to antibiotics resulting in colonized bacteria being up to 1000-fold times more resistant to antimicrobials than planktonic cells (Hoiby *et al.,*2010). Debridement removes ECM and eDNA to prevent recurrence of biofilms in the wound by removing the basis for nutrition and protection of the bacterial component of the biofilm (Hajska *et. al,* 2014). The immature biofilms that begin to reform following debridement are subsequently more susceptible to topical therapies. General principles described by Wolcott *et al.* in addressing wounds infected with biofilms include debridement with the goal to alter the wound bed anatomy by removing any devitalized or discolored tissue and all tissue surfaces that touch one another until normal bleeding tissue is encountered (Wolcott *et al.,* 2010). Application of topical treatments is then recommended within four hours following debridement prior to biofilm reformation (Roche *et al.,* 2012; Hajska *et al.,* 2014). An example of how biofilms may be successfully treated and how rapidly they reform in the absence of consistent treatment is daily removal of dental enamel plaque by regular tooth brushing (i.e., debridement) performed in combination with topical antiseptic mouthwashes, which are of minimal benefit without prior flossing and tooth brushing (Orsini *et al.,* 2017).

Biofilm debridement may be performed sharply (*e.g.,* scalpel blade), mechanically with gauze across the wound bed, or using water-jet irrigation or low-frequency ultrasonic debridement. It is recommended that horses be sedated, and the wound desensitized with local or regional anesthesia to facilitate procedures and reduce discomfort to the patient. In some cases, the initial debridement may be performed under general anesthesia if the wound is extensive or inaccessible or if dictated by the patient's temperament. When working with multi-drug resistant organisms or particularly when using pulsed water-jet irrigation, face protection or use of surgical masks during the debridement stage is recommended to protect again aerosolized organisms. Debridement and efforts to reduce biofilm reconstitution should be repeated daily to at least every other day for as long as necessary to resolve infection. Mature biofilms reform as rapidly as every 24 to 72 hours after debridement, resulting in a window of opportunity to impede regrowth in which topical therapies and bactericidal drugs can exert an enhanced effect. If improvement is not observed within three to four days of initiation of the multimodal therapeutic approach outlined or if response to therapy is less than anticipated, review of all aspects of the case is indicated. These may include repeated physical examination, bloodwork, evaluation of antibiotic suitability with repeated bacterial culture and sensitivity, and further debridement and exploration of the wound and potentially additional diagnostic imaging to evaluate for alternate reasons for delayed healing (*e.g.,* foreign material).

4.3. Topical therapies to prevent biofilm reformation - Reduction or prevention of biofilm reformation following debridement may be achieved in multiple ways. Topical antiseptic agents do not penetrate necrotic debris and have minimal effect to reduce bacterial populations deep in the wound bed or without debridement; therefore, they should generally be reserved for use on intact skin and in wound beds (Alves*et al.,* 2021). Examples of antiseptic agents contraindicated for use in biofilm associated wounds include alcohols, hydrogen peroxide, iodine, povidine-iodine, chlorhexidine, aluminum salts, boric acid, formaldehyde, hexachlorophene, hypoclorite, merthiolate, or permanganate. However, unlike antiseptics, topical antimicrobial agents can have minimal negative side effects on wound healing depending on the vehicle and dose used and provide efficacy against bacteria in the wound bed when administered following debridement and based on results of culture and sensitivity.

Surfactant dressings such as polyhexamethylene biguanide (PHMB) or polyhexanide can be used as adjunctive therapies in the early post-debridement period, as they reduce biofilm surface tension to facilitate degradation and removal (Palumbo *et. al.,* 2016; Percival *et al.,* 2019). Other topical dressings such as silver sulfadiazine (1%) or other silver impregnated wound dressings may be used in the early post-debridement stage to reduce biofilm reformation, particularly if bacterial culture and sensitivities to guide topical antimicrobial treatments are not available (Morones *et al.,* 2005; Fey *et al.,* 2010; Gunaskaran *et al.,* 2012). Silver works through interacting with ribosomes to suppress enzymatic expression and protein formation essential for ATP production (Yamanaka *et al.,*2005). The methods by which silver interacts with bacteria reduces formation of resistance and results in broad-spectrum antibacterial properties (Gunaskaran *et al.,* 2012). In addition, silver enhances re-epithelialization, angiogenesis, deposition of collagen fibers, and myofibroblast distinction from fibroblasts prompting wound contraction (Toczek *et. al.,* 2022). Manuka honey also exhibits antimicrobial properties due to high methylglyoxal and leptosperin content and may be used as an adjunctive topical antimicrobial therapy against a variety of bacterial species with minimal host cytotoxicity (Molan and Rhodes, 2015; Liu *et al.,* 2017). Finally, topical application of plasma (autologous natural plasma or hyperimmune plasma to target specific organisms) may provide additional benefit as a topical therapy as plasma inhibits bacterial adhesion and growth (Felts*et al.,* 2000; Bauer *et al.,* 2004; Lopez *et al.,*2014).

4.4. Antimicrobial guidelines – In general, contaminated wounds including those with suspected biofilm involvement are more appropriately treated with bactericidal versus bacteriostatic antimicrobial agents. Although ideally dictated by culture and sensitivity findings, broad-spectrum antimicrobial therapy is generally instituted initially with commonly administered agents include penicillins, cephalosporins, aminoglycosides, quinolones, metronidazole and rifampin (Orsini *et al.,* 2017). Commonly used initial combinations include pencillin G (crystalline or procaine penicillin) or a cephalosporin (cefazolin or ceftiofur) and an aminoglycoside such as gentamicin. Collection of a separate sample to perform in-house point-of-care Gram staining may help to guide interim antimicrobial therapy in lieu of culture and sensitivity findings. In treatment of distal limb wounds, antimicrobials can also be delivered via regional limb perfusion. Antimicrobial concentrations delivered locally are greatest immediately following biofilm degradation so timing of perfusion to directly follow debridement may improve outcomes although further investigation is indicated. Finally, repeated culture and sensitivity is also generally considered indicated in cases where response is less than anticipated, signs of infection recur following discontinuation of antimicrobials, if the infection is polymicrobial or multidrug resistant, or during periods of prolonged antimicrobial administration.

4.5. Other considerations - Limitations of current laboratory testing and definitive clinical signs indicating biofilm presence make it impossible to objectively determine whether biofilms have been eradicated from a wound. Further investigation of stall-side testing techniques to identify biofilm presence may enhance monitoring techniques in the future; however, currently, monitoring of clinical progression with reduced exudate and slough remains the most effective method to determine response to treatment and biofilm resolution (Leaper*et al.,* 2012). However, despite appropriate treatment, biofilms associated with orthopedic implants or other foreign devices frequently necessitate removal for resolution (Richardson and Stewart 2019). In some cases, infection can be controlled temporarily through a combination of systemic and local antimicrobial therapy until fracture or arthrodesis consolidation has occurred (Wu *et al., 2014*). If cases with both infection and instability, implants may be removed and replaced, or cleaned, sonicated, and reimplanted

using new orthopedic screws when financially feasible. Alternatively, internal implants may be removed, and cases managed with a transfixation pin cast or other external fixator. The fracture site and surrounding tissues should be debrided and lavaged, and previous screw holes and the surrounding region may be treated locally with antibiotic eluting materials. In general, when communicating with clients about the cost of care in biofilm-associated wounds, it is recommended to emphasize that the greater expense incurred in the earlier stages of wound management typically reduces duration of therapy and costs overall in treatment long-term (Orsini *et al.,* 2017). Clinical case examples where wounds with biofilms were successfully treated are provided and summarized in **Figures 1 and 2** .

Future directions in diagnostic techniques and treatment strategies for biofilms

Novel techniques to reduce infection burden associated with biofilms are currently being investigated and further developed. Methods described include further investigation of surfactant-based agents, cellular therapeutic options (*e.g.,* platelet rich plasma lysates, mesenchymal stromal cells), quorum-sensing inhibitors (RNAIII inhibiting peptide), hydrophobic polycationic or sol gel coatings, bacteriophage therapies (antibacterial viruses), antimicrobial peptides, ultraviolet light, low-voltage pulsed electrical fields, acetylsalicylic acid, xylitol, dispersin B, gallium, or antimicrobial tethering (Tiller*et al.,* 2001; Levy *et al.,* 2004; Nablo *et al.,*2005; Balaban *et al.,* 2005; Williams and Hare 2011; Stewart*et al.,* 2012; Schaer *et al.,* 2012; Barsotti *et al.,*2013; Spaas *et al.,* 2013; Bussche *et al.,* 2015; Mohammed*et al.,* 2016; Grassi *et al.,* 2017; Orsini *et al.,*2017; Hans *et al.,* 2019; Gilbertie *et al.,* 2021; Gordon*et al.,* 2021). In addition, future diagnostic tests may be more effective at definitively identifying the presence and location of biofilms within a wound bed to guide more patient-specific treatment strategies. For example, a stall-side or patient-side test to quantify wound bed protease activity could be one method to indirectly quantify and longitudinally evaluate the amount of residual biofilm in a wound, as protease activity correlates generally to the amount of viable or active biofilm (Leid *et al.,* 2002). Further evaluation of methods to improve detection of biofilms, monitoring of treatment efficacy, and overall management of biofilms in case-controlled studies and randomized controlled clinical trials in warranted.

Conclusions

The recognition that most chronic wounds in equine practice involve pathogenic bacterial biofilms is key to successful treatment. Clinical indications that biofilms are present in the wound bed include wounds that remain in a chronic inflammatory state recalcitrant to standard therapies, excessive exudate/moisture, poor quality local granulation tissue, other common indications of infection (heat, swelling, pain), and/or negative bacterial culture results despite clinical suspicion of infection. Biofilm-based wound care strategies emphasize repeated debridement and lavage combined with topical surfactants or antimicrobials applied within four hours of debridement that have minimal local cytotoxicity to host tissues. Finally, improved diagnostic tools to detect biofilms and monitor response to treatment as well as adjunctive treatments may facilitate improved outcomes in the future.

Authors' declaration of interests: No conflicts of interest have been declared.

Ethical animal research: Review of current literature; not applicable.

Source of funding: None.

Authorship: All authors contributed to conception and design, acquisition of data, drafting and final approval of the manuscript.

Tables

Table 1: Indirect clinical indications of wound biofilm. (Metcalf et al, 2014; Wolcott et al. 2008)

Clinical Observation	Biofilm Expla
Excessive moisture associated with wound	Bacteria in biofi
Autograft or allograft fails on wounds	Applying tissue

Clinical Observation	Biofilm Explan
Poor quality granulation tissue (e.g. hypergranular, friable)	Biofilm presence
Indications of local infection (swelling, sensitivity, redness, heat)	Biofilms promote
History of persistent or recurrent infection despite antimicrobial therapy	Biofilm bacterial
Negative culture results despite clinical suspicion of infection or signs of bacterial colonization	Biofilm bacteria
Wound remains in chronic inflammatory state and recalcitrant to therapy despite addressing comorbities	Biofilms are resis

Table 2: Key statements on biofilms in wounds (Schulz et al, 2017)

Biofilms are present in most chronic wounds and are likely to be present on both the surface and deeper wound layers but n
Biofilms are difficult to visualize macroscopically, and exudate, slough or debris may be mistaken for biofilms.
Wounds that contain biofilms may not be identified, leading to ineffective treatment and delayed healing.
Important clinical indications that a wound likely contains a biofilm include lack of response to treatment with antibiotics o
Debridement is one of the most important treatment strategies against biofilms; however, biofilms reform rapidly, so debride

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Figures

Figure 1: Case example 1. 7-day old Warmblood foal was presented for acute swelling of the right tarsus. An approximately 2cm x 2cm abrasion was noted over the lateral aspect of the tarsus with moderate periarticular edema was appreciated. Radiographs of the tarsus revealed no significant abnormal findings and fluid analysis of synovial fluid from the tibiotarsal joint was within normal limits. Ultrasound of the umbilicus, abdomen and thorax revealed no significant abnormal findings. Within 3 days following presentation, the colt developed marked cellulitis of the right hindlimb which was initially with a compression/sweat bandage, and intravenous antibiotic therapy (amikacin, potassium penicillin) and anti-inflammatories (flunixin meglumine). Ultrasound of the limb revealed a suspected extra-articular subcutaneous abscess forming near the level of the distal intertarsal and tarsometatarsal joints. Five days after initial presentation, strike-through was noted diffusely throughout the bandaged limb and when the bandage was removed a generalized necrotic open wound extending from the level of the tarsus to the fetlock was appreciated. Culture and sensitivity yielded *Citrobacter, Enterobacter* and*Staphylococcus aureus* . Based on sensitivity results, antibiotics were transitioned to ceftiofur sodium. Repeated daily to every other day topical debridement, lavage, and wound care with kerlix AMD surfactant impregnated gauze dressings were performed. Approximately 3 weeks after initial presentation, the wound bed was deemed healthy enough for skin grafting. Full thickness mesh graft obtained from the ventral abdomen was performed under general anesthesia. Two weeks later he received a second mesh graft to cover the remaining area of the wound, obtained from his pectoral region. The tissue here obtained from the ventral abdomen was a different color but overall, the result was considered a good cosmetic outcome. The foal was dismissed to the care of his owners after approximately 5 weeks hospitalization and continued to do well at home with approximately four years follow-up to date.

Figure 2: Case example 2. A 14-year-old Quarter Horse gelding was presented for evaluation of a 5-month-old wound on the dorsal aspect of the tarsus, sustained the previous summer on barbed wire fence. He had been initially treated with trimethoprim sulfamethoxazole antibiotics. Radiographs of the tarsus were performed which revealed no significant abnormal findings and he was turned out on pasture. When the wound continued not to heal, he was brought to Colorado State University Veterinary Teaching Hospital for further evaluation. Radiographs at that time revealed no sequestrum or osseous involvement. The gelding

was induced under general anesthesia and the wound was sharply debrided and lavaged. He was maintained on antibiotics with bandaging in kerlix AMD and splinting to minimize motion through the tibiotarsal joint. A second debridement was performed approximately 3 weeks following the first and samples collected for culture. He was transitioned from trimethoprim sulfamethoxazole antibiotics to enrofloxacin based on culture and sensitivity results. Additionally, he was enrolled as a pilot case in a study evaluating the effect of mesenchymal stromal cell therapy to improve wound healing. The lateral half of the wound bed was treated with 3 doses of intralesional doses of 30 million stem cells. At approximately 3 months following initial presentation, the wound bed was considered healthy enough to support a graft. A pinch skin graft was performed from skin obtained from the left ventral abdomen. Interestingly, although the wound did fully epithelialize by 10 months following initial presentation, the lateral aspect of the wound that had been injected with stromal cells did so more rapidly, indicating that the antimicrobial and immunomodulatory properties of MSC may help to accelerate healing and warrants further investigation.

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