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Microbiology of equine wounds and evidence of bacterial biofilms

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Abstract

Horse wounds have a high risk of becoming infected due to their environment. Infected wounds harbour diverse populations of microorganisms, however in some cases these microorganisms can be difficult to identify and fail to respond to antibiotic treatment, resulting in chronic non-healing wounds. In human wounds this has been attributed to the ability of bacteria to survive in a biofilm phenotypic state. Biofilms are known to delay wound healing, principally due to their recalcitrance towards antimicrobial therapies and components of the innate immune response. This study describes the presence of bacterial biofilms within equine wounds. Thirteen eight millimetre diameter tissue samples were

collected from (n=18) chronic wounds. Following histological staining, samples were observed for evidence of biofilms. Fifty one wounds and control skin sites were sampled using sterile swabs. Control skin sites were on the uninjured side of the horse at the same anatomical location as the wound. The isolated bacteria were cultured aerobically and anaerobically. The biofilm forming potential of all the isolated bacteria was determined using a standard crystal violet microtitre plate assay. Stained tissue samples provided evidence of biofilms within 61.5% (8 out of 13) equine wounds. In total 340 bacterial isolates were identified from all the equine wound and skin samples. *Pseudomonas aeruginosa* and *Enterococcus faecium* were the most predominantly isolated bacterial species from equine wound and skin samples respectively. *Staphylococcus* was the most commonly isolated genus in both environments. Bacteria cultured from chronic and acute wounds showed significantly ($P<0.05$) higher biofilm forming potential than bacteria isolated from skin. This paper highlights preliminary evidence supporting the presence of biofilms and a high microbial diversity in equine chronic wounds. The presence of biofilms in equine wounds partly explains the reluctance of many lower limb wounds to heal. Non-healing limb wounds in horses are a well documented welfare and economic concern. This knowledge can be used to shape future treatments in order to increase the healing rate and decrease the costs and suffering associate with equine wounds.

Keywords

biofilm · equine · wound · bacteria · microbiota · identification

Introduction

The incidence and prevalence of trauma wounds in horses is considered to be high (Singer et al., 2003) and when these wounds fail to heal they are referred to as chronic wounds (Adam and Southwood, 2006). Human and equine chronic wounds are considered to be pathologically similar (Cochrane, 2003) but unlike human chronic wounds the microbiology of equine wounds has not been studied in any great detail, either in terms of the number of inhabiting bacteria, the types of inhabiting bacteria or the ability of bacteria to form protective biofilms. In a number of human wound studies there is significant evidence suggesting that chronic wounds have a delayed healing response due to the presence of a polymicrobial biofilm (Davis et al., 2008). In these cases, wounds may appear to be uninfected, but resist healing due to a persistent bacterial population residing within protective biofilm.

A biofilm is the name given to a community of microorganisms attached to a surface, or each other, and encased within a three dimensional matrix of extracellular polymeric substances (EPS) (Mertz, 2003). Biofilm formation has been linked to an increased inherent resistance to antimicrobial agents and the host's immune response (Stewart and Costerton, 2001). For example *Staphylococcus aureus* when growing within a biofilm has been reported to be up to 100 times more resistant to antimicrobials when compared to their free floating or planktonic counterparts (Leid et al., 2000). Consequently biofilms are known to be a causative agent in persistent mammalian infections (Costerton et al., 2003) including reoccurring leg ulcers (Bjarnsholt et al., 2008). Recently, preliminary evidence proposing the presence of biofilms in chronic equine wounds was reported (Freeman et al., 2009).

69

70 The purpose of this study was two-fold. Firstly it aimed to highlight evidence that biofilms
71 exist in equine wounds; by examining tissue sections for evidence of biofilms and by
72 comparing the virulence of bacteria isolated from chronic and acute equine wounds, and from
73 equine skin. Virulence was assessed by determining the biofilm forming potential (BFP) of
74 the isolates. Using skin cultures as a control, the study also aimed to investigate the microbial
75 richness of equine wounds, in order to highlight differences in the species that were prevalent
76 in chronic wounds, acute wounds and equine skin.

77

78 **Material and methods**

79

80 **Experimental design**

81

82 Between November 2007 and May 2008, 51 of the horses and ponies at the Philip
83 Leverhulme Equine Hospital (PLEH), Neston, UK that presented with acute or chronic
84 wounds were selected for this study. All 41 trauma wound cases admitted to PLEH within the
85 study period were included in the study. Ten healing surgical wound cases were randomly
86 selected for inclusion within the study in order to present a wound control group. The
87 selection criteria for surgical wounds entailed sampling 1 clean surgical wound for every 4
88 wound cases. The surgical case was selected on the basis of being admitted to the hospital at
89 the same time as the fourth wound case (or every 4 thereafter). Breeds incorporated into the
90 study included 16 Thoroughbred/Thoroughbred cross, eight Cobs, four Arab/Anglo Arabs,
91 four Warm bloods, four Welsh ponies one Hunter type, one Clydesdale, one American
92 quarter horse, one shire and nine horses and two ponies of unknown breed. The horses were
93 aged between two and 22. There were 33 colts/ geldings and 18 fillies/ mares. Informed

consent was obtained from the owners of the animals participating in this study. Prior to sample collection all the horses included in this study had received systemic antibiotics, topical antibiotics or both; however information regarding the types and duration of antibiotic treatment were not recorded. No antimicrobial dressings such as Silver dressings had been used prior to sample collection. Of the 51 wounds analysed, 10 were of surgical origin, and 41 were the result of trauma. Surgical wounds were the result of elective surgery and, trauma wounds were the result of an injury caused by a foreign object. The wounds were categorised as chronic or acute by the attending veterinary surgeon. Acute wounds (including all surgical wounds) were seen to progress at a normal rate through each stage of the healing process, and did not show signs of local or systemic infection. Wounds were classified as chronic if the case description involved a non-healing wound, or if the wound showed two or more chronic signs, including; bright red colouration, slimy surface, discharge, fetid odour, and/or a lack of healthy granulation tissue (Goodrich, 2006). Of the 51 wounds sampled, 33 were categorised as acute and 18 as chronic. A skin swab was taken from uninjured skin at the same anatomical site of the horse as the wound, but on the uninjured side of the horse, for example if the wound site was the left front carpus, the skin site was the right front carpus. Wounds included in this study were a mixture of thoracic and limb wounds. All swab samples were analysed microbiologically for aerobic and anaerobic bacteria, using traditional microbiological techniques as described below. The biofilm forming potential for each isolate was determined using crystal violet stained microtitre plate assays. As part of the normal wound treatment, debrided tissue samples that would usually be disposed of as surgical waste were removed from the edge of 5 wounds as described below. These samples underwent histological staining in order to look for evidence of bacterial biofilms.

Tissue sampling

119

120 As part of the normal wound debridement treatment, tissue samples that were classified as
 121 surgical waste were collected from the edge of the wound. Samples of the debrided tissue
 122 were sectioned using a disposable eight mm punch biopsy (Amcor flexible, UK). The tissue
 123 was then immediately stored in formalin (VWR, Leicestershire, UK) for 24h, and then
 124 sectioned and stained with three histological stains; Gram stain, haematoxylin and eosin (H
 125 and E) and Masson's trichrome (MT) stains. All Stains were made from individual reagents,
 126 sourced from H.D Supplies, Buckingham, UK, V.W.R International, Leicestershire, UK,
 127 Fisher Scientific UK Ltd Leicestershire, UK and Sigma-Aldrich Company Ltd, Dorset, UK.
 128 The stained samples were examined under light microscopy (Nikon Eclipse 80i with digital
 129 sight capture) at a magnification of x1000.

130

131 **Bacterial sample collection using swabs**

132

133 Standard microbiological protocols as described below were utilised for the sampling of the
 134 wound and skin sites. All sampling procedures were performed within one to three days of
 135 the horse or pony being admitted to PLEH. Wound and skin sampling sites were washed with
 136 sterile saline prior to swabbing in order to remove surface debris. Swabbing involved holding
 137 the sterile cotton tipped swab between the thumb and fore finger and rubbing it across a 1cm²
 138 area for five seconds, as described in the Levine technique (Levine et al., 1976). Surgical
 139 swabs were taken from the surface of the wound which had been closed by primary intention.
 140 All wound and skin sites were swabbed in triplicate. The first swab was stored in Stuart's
 141 transport medium (Oxoid Limited, Hampshire, UK) and used to isolate aerobic bacteria, the

second was added to Robertson's Cooked Meat Broth (Oxoid Limited, Hampshire, UK), for the enrichment of anaerobic bacteria, and the third was stored in an eppendorf tube and frozen at -20°C for molecular analysis.

Bacterial isolation and identification

Aerobic swabs were stored at 4°C and processed within 24h. Aerobic samples were inoculated onto the enrichment agar Tryptone soy agar (TSA) and the selective agars; MaConkey agar (MCA), Columbian blood agar (CBA), and Cysteine lactose electrolyte deficient (CLED) agar (Lab M, Bury, UK). All agar plates were incubated for 24 hours at 37°C (+/-2°C). Swabs which had been stored in Robertson's cooked meat broth were incubated at 37°C for 24 hours, and then at room temperature for 1 week. At 48 hour intervals samples of this media were added to fastidious anaerobic agar (FAA) (Lab M, Bury, UK), for the culture of anaerobic bacteria. The prolonged incubation period was used to enhance the recovery of slow growing anaerobic species. Isolates grown in anaerobic conditions were also sub-cultured onto CBA and incubated overnight in aerobic conditions at 37 °C. Isolates that grew in both aerobic and anaerobic conditions were identified following the aerobic protocol.

Following incubation, all the phenotypically distinct bacterial colonies were sub cultured onto TSA (aerobic isolates) or FAA (anaerobic isolates). Pure bacterial colonies were identified using Gram staining, oxidase testing, catalase testing, coagulase testing and biochemical analysis. API identification strips (Oxoid Ltd, Hampshire, UK), Vitek identification cards

(BioMerieux, Hampshire, UK) and the Microseq 3130 Microbial Identification System (Applied Biosystems, UK) were utilised for further species identification.

Microtitre plate assay for the assessment of biofilm formation

The biofilm forming potential of all the bacteria identified throughout this study was measured using a published method (Wakimoto et al., 2004). In brief, colonies of individual purified bacteria were inoculated into Muller Hinton Broth (MHB), and incubated overnight at 37°C in an orbital incubator (50rpm). Following incubation, all the bacterial suspensions were adjusted to 10^8 cfu ml⁻¹ using sterile MHB. The concentration was confirmed using a 0.5 McFarland standard (Stepanović et al., 2007). Then 250µl of each inoculum was transferred into 3 wells of the Costar 96 well microtiter plates (Corning Costar, NY, USA). The 96 well plates were incubated at 37°C for 24 h at 50 rpm in an orbital incubator. Following incubation, each well was washed (x3) with sterile saline. Attached bacteria were then fixed in 96% pure ethanol and then stained with 2% crystal violet. Excess crystal violet was then aspirated from the wells, and the wells were washed (x3) with sterile distilled water to remove all excess dye and planktonic bacteria. Each well was then filled with 200µl of 33% glacial acetic acid to remove the stained biofilm. The optical density (OD) of each well was measured at 570nm using an Opsys MR microtiter plate reader (DYNEX Technologies, Worthing, UK). The average OD of the control wells (containing MHB only) was subtracted from the OD of the test wells. All isolates and controls were tested in triplicate.

Statistical analysis

A Wilcoxon signed rank test was used to investigate differences in the genus and species of bacteria present at wound sites compared to control sites. A Fisher's exact Chi Squared test was used to assess the different kinds of bacteria found in chronic wounds compared to acute wounds and in surgical wounds compared to traumatic wounds. A Mann Whitney U test was used to compare the presence of different bacterial genus at each sample site, and also to compare the biofilm forming ability of each bacterial genus, and the biofilms forming ability of the bacteria isolated from different wound types. All statistical analysis was carried out using the statistical package Minitab 15.

Results

Tissue samples

Bacterial clustering was seen in the stained tissue samples, suggesting evidence of a bacterial biofilm, based on published observations and biomarkers (Parsek and Singh, 2003). Infected tissue contained extensive 'thumb-print' like aggregates and microcolonies of Gram positive cocci and Gram negative rods, at the epidermal surface of the debrided tissue (Figure 1). Although Gram positive and Gram negative bacteria occupied the same tissue regions, more often than not, they appeared to cluster in discrete segregated microcolonies. The staining also highlighted extensive evidence of inflammatory cells throughout the chronic wound tissue. In un-infected tissue, little or no evidence of bacteria was seen (Figure 2). This was supported by a culture negative swab sample.

Bacterial isolation

From 51 equine skin and wound swab samples, 340 bacterial isolates were cultured (Table 2) and 338 were identified to the species level. The most common bacterial species isolated from wounds was *P. aeruginosa* (5.6%) and the most commonly isolated species from skin was *E. faecium* (5.6%). *Staphylococcus sp* were isolated more often than most other genus ($P<0.05$) and there was significant difference in the genera isolated from wound and skin sites ($P<0.05$).

The wound swabs were further divided into wounds that were the result of surgery or a traumatic incident, and wounds that were deemed chronic or acute (Figure 3). The average number of bacterial species identified from trauma wound swabs was 3.02 ± 1.65 (range 0-8) and the average number of species per surgical wound swab was 3.8 ± 3.82 (range 1-14). Fourteen isolates were identified from only one surgical wound. At a genus level, the number of isolates identified per swab for trauma and surgical wounds was 2.51 ± 1.36 (range 0-6) and 2.8 ± 1.87 (range 1-7) respectively. Again the mean number of surgical wound isolates obtained per swab was increased due to the inclusion of the surgical wound containing 14 isolates.

The species most often isolated from traumatic wound swabs and surgical swabs was *P. aeruginosa*, where *P. aeruginosa* made up 4.84% and 7.89% respectively, of the total identified species. However the most commonly isolated genus in both traumatic and surgical

wounds was *Staphylococcus*, which respectively made up 22.58% and 18.42% of the total identified bacterial load. The *Staphylococcus* species cultured from equine wounds included; *S. aureus*, *S. auricularis*, *S. epidermidis*, *S. xylosus*, *S. Sciuri*, *S. simulans*, *S. warneri*, *S. equorum*, *S. hominis*, and *S. pasteuuri*. *Proteus* sp, *Acinetobacter* sp, *Salmonella* sp, *Alcaligenes* sp, *Propionibacterium* sp, *Bordetella* sp, *Cellulomonas* sp, were found exclusively in trauma wounds. Furthermore the prevalence of *Staphylococcus* sp in traumatic wounds was greater than the prevalence of *Micrococcus* sp, *Enterococcus* sp, *E. coli* sp, *Enterobacter* sp, *Alcaligenes* sp, *Clostridium* sp, *Propionibacterium* sp, *Bordetella* sp, and *Cellulomonas* sp ($P < 0.05$).

Of the 162 identified wound isolates, 32.72% were of chronic wound origin, and 67.28% were isolated from acute wounds. *Pseudomonas* spp were significantly ($P < 0.05$) more prevalent in chronic wounds than acute wounds. This kind of variation may be occurring between other genus such as *Proteus* and *Enterobacter*, but this difference was not significant with the current sample size. The genera *Aerococcus*, *Micrococcus*, *Alcaligenes*, and isolates that could not be identified, were isolated exclusively from acute wounds, but no significant difference was seen in the data (Figure 4).

In chronic wounds, *Pseudomonas* spp were identified significantly more often than *Aerococcus* spp, *Micrococcus* spp, *Pasturella* spp, *Corynebacterium* spp, *Alcaligenes* spp, *Bordetella* spp, and *Cellulomonas* spp ($P < 0.01$) and to a lesser extent ($P < 0.05$), *Bacillus* spp, *Acinetobacter* spp, *Salmonella* spp, *E. coli*, *Clostridium* spp. and *Propionibacterium* spp. No significant difference was seen in the isolation prevalence of the other identified bacterial

genus within chronic wounds, and there was no significant difference in the prevalence of isolates cultured from acute wounds.

Obligate anaerobic isolates were recovered from swabs and made up 6.8% and 7.3 % of the total wound and skin isolates respectively. There was no significant difference in the amount of times obligate anaerobes were identified from wound and skin swabs or between the anaerobic recovery rate from the swabs used to sample different wound types.

Microtitre plate assay for the assessment of biofilm formation

The species *Aerococcus viridans* 3, *S. warneri*, *B. licheniformis*, *B. pumilus*, and *Acinetobacter* spp. all showed significantly stronger biofilm formation when they were isolated from wounds compared to skin ($P < 0.05$). *Enterococcus durans* was the only species to show significantly higher biofilm formation (BFP) when isolated from skin than from wounds ($P < 0.05$). Collectively the bacteria isolated from wounds displayed a significantly ($P < 0.05$) higher BFP than those isolated from skin. Wound isolates had an average BFP value of 0.50 ± 0.35 and skin isolates had an average value of 0.32 ± 0.30 . An optical density above 0.2 was indicative of bacteria that showed a level of adherence linked to biofilm formation (Table 1) (Stepanovic et al., 1999).

At a genus level, the wound isolates *Pseudomonas*, *Proteus*, *Bacillus* and *Aerococcus* displayed significantly stronger attachment, indicative of biofilm formation, than the equivalent skin isolates ($P < 0.05$) (Figure 5). *Pseudomonas* and *Proteus* spp. wound isolates displayed strong adherence ($OD > 0.4$) and *Bacillus* and *Aerococcus* spp. displayed moderate

adherence (OD between 0.2 and 0.4). The genus *Moraxella* produced the highest wound isolate OD (OD = 2.17 ± 1.33), but the largest OD recorded was from skin isolates of *Myroides* bacteria (OD = 2.46 ± 0.05). These values were suggestive of extremely strong adherence and thus a strong BFP. The genera *Burkholderia* sp, *Shigella* sp, *Yersinia* sp, *Rhizobium* sp, *Sphingomonas* sp, *Pasteurella* sp and *Aeromonas* sp were isolated exclusively from skin swabs. These isolates had ODs of between 0.01 and 0.37.

The BFP of Gram negative trauma wound isolates was significantly ($P < 0.01$) greater than those of Gram negative surgical wound isolates. However there was no significant difference in the BFP of Gram positive isolates sampled from trauma and surgical wounds. At a species level the species *S. epidermidis*, *S. xylosum*, *B. subtilis*, *Enterococcus faecalis* and *Enterobacter cloacae* all displayed a stronger BFP when isolated from surgical wounds than when they were isolated from trauma wounds. Similarly, *Enterobacter cloacae* and *Bordetella pertussis* both displayed a stronger biofilm forming potential when they were isolated from acute wounds compared to chronic wounds.

There were significant differences ($P < 0.05$) in the BFP of different isolates within trauma wounds. The Gram positive isolates *Staphylococcus* spp. *Micrococcus* spp. *Bacillus* spp. and *Enterococcus* spp. and the Gram negative *Pseudomonas* isolates all showed significantly ($P < 0.05$) greater attachment than *Enterobacter* spp.

Discussion

Tissue samples taken from chronic equine wounds displayed morphologically similar bacterial clustering to those seen in human biofilm infected wounds (Costerton et al., 1994). Bacteria displayed a greater BFP when they were isolated from wounds compared to skin, suggesting that equine wounds harbour bacterial pathogens which display the biofilm phenotype more commonly than the equivalent skin isolates. Chronic equine wounds did not harbour bacteria that displayed a stronger BFP than acute wound. An explanation for this is that culture results are heavily weighted towards easily cultured organisms (Dowd et al., 2008). If biofilm forming isolates in chronic wounds were encased within a bacterial biofilm, as suggested by the tissue sections, then the chance of a false negative culture may have been increased (Costerton et al., 2003).

Equine wounds were polymicrobial and contained pathogens which were significantly more prevalent in chronic wounds than acute wounds. *Aerococcus viridians*, *S. warneri* and *Acinetobacter* Spp. demonstrated greater BFP when they were isolated from equine wounds compared to when they were isolated from equine skin. In human studies *Aerococcus viridians* has been linked to leg ulcers (Cooper et al., 2009) and infected surgical wounds (Huljev and Kucisec-Tepes, 2005), *S. warneri* has been linked to osteomyelitis (Bryan et al., 1987) and endocarditis (Wood et al., 1989) infections, and *Acinetobacter* Spp. have been repeatedly linked to chronic wounds (Gjodsbol et al., 2006; Hill et al., 2003; James et al., 2008). These pathogens may have contributed to the healing retardation of the chronic wounds in this study. Interestingly *B. licheniformis* and *B. pumilus* also demonstrated a greater BFP when isolated from wound sites compared to skin sites but previous literature has highlighted their ability to disrupt *S. aureus* (Rivardo et al., 2009) and *Pseudomonas* (Nithya et al., 2010) biofilms.

325

326 This study used bacterial attachment as a measure of BFP. Most of the species displayed
 327 some ability to attach to the polystyrene surface suggesting that the adaptive mechanism of
 328 biofilm formation is exhibited to some degree by the majority of wound colonising bacteria.
 329 The degree to which different bacterial species attached to the polystyrene surface of the
 330 microtitre plate wells was variable, reflecting the fact that not all organisms have an equal
 331 ability to attach to polystyrene (Harrison et al., 2010). As such, *in vitro* attachment may not
 332 accurately reflect *in vivo* attachment. Variations in the *in vivo* attachment are also likely to
 333 reflect the synergistic interactions of the colonising/ infecting populations (Cooper et al.,
 334 2009). All the horses in this study were receiving antibiotic treatment. The antibiotic
 335 challenge may have favoured the biofilm forming phenotype (Dancer, 2008) however this
 336 accurately reflects the clinical situation in which biofilms are problematic.

337

338 The OD of bacteria attached to the 96 well plates, ranged from 0 to 2.46. These values
 339 concurred with previously presented data for 24 hour biofilms (Djordjevic et al., 2002).
 340 However the range in this study was slightly larger than the range reported in some previous
 341 studies. Previous data was obtained for single species biofilms for example *Staphylococcus*
 342 biofilms, *E coli* biofilms and *Listeria monocytogenes* biofilms were assessed by Stepanovic *et*
 343 *al* (1999), Wakimoto *et al* (2004) and Djordevic *et al* (2002) respectively. The multispecies
 344 nature of this investigation explains the large range of OD values obtained in this study.
 345 Knowledge about the BFP of different bacterial species allows researchers and clinicians to
 346 recognize the isolates most likely to form a biofilm. Further work would be required in order
 347 to advise appropriate treatment and prevention strategies for the removal of bacterial biofilms
 348 from equine wounds, however if their aetiology is assumed to be similar to that of human

diabetic ulcers, thorough debridement coupled with appropriate antibiotic treatment would be proposed (Davis et al., 2006).

The isolation of multiple bacterial species per swab supports the theory that equine *in vivo* wound environments harbour a polymicrobial community of microorganisms (Freeman et al., 2009). However it has been well reported that the culture of isolates using a swabbing technique can results in an overrepresentation of surface bacteria such as *S. aureus* and an underrepresentation of isolates that reside more deeply within the wound (Fazli et al., 2009). Despite this observation, these authors deemed swabbing to be the most appropriate sampling technique for this study due to its un-invasive nature. In human diabetic foot ulcers the synergistic interactions of bacteria are thought to promote an environment that supports the development and maintenance of a chronic wound (Dowd et al., 2008). The evidence presented in this paper suggests that this scenario is also likely in chronic equine wounds, adding weight to the theory that equine wounds provide a good model for human chronic wounds (Cochrane, 2003).

Microbial diversity varies between acute and chronic wounds, for example *Pseudomonas* spp. were more common in chronic wounds than acute wounds ($P < 0.05$). The opportunistic pathogenesis of isolates such as *Pseudomonas* have been well documented (MacDonald et al., 1994). The possibility that opportunistic pathogens are resisting standard treatments by residing in a biofilm is of great medical importance, in terms of individual cases and antibiotic usage in veterinary medicine (Van den Eede et al., 2009). *Staphylococcus* and *Enterococcus* spp. were also seen to colonise chronic wounds, supporting earlier work which

highlighted their presence in traumatic (Orsini et al., 2004) and in surgical equine wounds (MacDonald et al., 1994).

Recognising and understanding biofilms in equine wounds would help to guide appropriate management strategies for the treatment of recalcitrant wounds in horses. The use of appropriate anti-biofilm therapies in equine wounds may help to improve equine welfare by increasing healing rates and help to decrease the emergence of antibiotic resistant bacterial strains in response to inappropriate and ineffective antibiotics.

Conclusion

This paper has highlighted preliminary evidence supporting the presence of biofilms in equine chronic wounds. The presence of these biofilms could partly explain the reluctance of many lower limb equine wounds to heal in a timely manner, despite the use of traditional wound management strategies.

Multiple bacterial species can be isolated from equine skin and wounds providing evidence of a microbial community that is not removed by standard wound cleansing. Although wound colonisation does not necessarily prevent wound closure, the presence of multiple bacterial species capable of biofilm formation suggests that these bacteria may be surviving and proliferating within a biofilm and subsequently hindering wound closure.

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Table 1. The interpretation of optical density values. These values were obtained from bacteria which had attached to the 96 well plate and been stained with crystal violet (Stepanovic *et al*, 1999)

Table 2. The number of times each genus was identified from wound and skin swabs. The number shown in brackets is the total number of species identified in that genus. When a genus could not be identified to the species level it was reported as Spp. Spp was counted as 1 species.

Figure 1. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing evidence of Gram negative (arrow heads) and Gram positive (long arrows) and individual colonies (elbow arrows). The epidermal edge of the wound is to the left of the image. (x1000 magnification).

Figure 2. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing an area of uninfected tissue. The epidermal edge of the wound is visible on the bottom right of the image. (x1000 magnification) (Bar +100µm).

Figure 3. Hierarchical flow diagram showing the number of each wound type included in the study. Control swabs were also taken from the uninjured skin of all the 51 cases.

516 **Figure 4.** Percentage representation of bacterial genera isolated from chronic wounds (black
517 bar) and acute wounds (grey bar) (n=51). * - significant difference between chronic and acute
518 wounds $P < 0.05$.

519

520 **Figure 5.** A comparison between the OD of wound (Black bars) and Skin (Grey bars)
521 isolates. The upper graph shows Gram positive isolates and the lower graph shows Gram
522 negative isolates. * show significant differences between wound and skin OD values * -
523 $P < 0.05$, ** - $P < 0.01$.

The interpretation of the optical density values obtained from the crystal violet assays

Optical Density	Interpretation
0	Non-adherent
≤ 0.2	Weakly adherent
$> 0.2 - \leq 0.4$	Moderately adherent
> 0.4	Strongly adherent

Table 1. The interpretation of OD values obtained from the attachment of bacteria grown on 96 well plates as part of a crystal violet assay. Stepanovic *et al*, 1999.

Genus	Wound samples (N = 51)		Skin samples (N = 51)	
	Number of times the genus	Number of species	Number of times the genus was	Number of species
	was isolated	represented	isolated	represented
<i>Staphylococcus</i> (14)	35	10	34	14
<i>Bacillus</i> (8)	16	6	17	7
<i>Enterococcus</i> (6)	16	5	16	5
<i>Pseudomonas</i> (6)	15	5	10	5
<i>Uncultured bacterium</i>	13	*	12	*
<i>Clostridium</i> (3)	6	2	11	3
<i>Escherichia</i> (1)	4	1	7	1
<i>Acinetobacter</i> (4)	5	2	5	4
<i>Propionibacterium</i> (1)	5	1	4	1
<i>Micrococcus</i> (2)	3	2	4	1
<i>Proteus</i> (2)	4	2	2	1
<i>Enterobacter</i> (2)	4	1	2	1
<i>Bordetella</i> (1)	4	1	2	1

<i>Aerococcus</i> (4)	3	2	2	2
<i>Pasteurella</i> (2)	0	*	5	2
<i>Cellulomonas</i> (1)	2	1	3	1
<i>Aeromonas</i> (1)	0	*	4	1
<i>Salmonella</i> (2)	2	1	2	2
<i>Corynebacterium</i> (1)	0	0	4	1
<i>Alcaligenes</i> (2)	2	2	2	2
Others (33)	23	18	27	22
No Identification	1	*	2	*

Table 2. The number of times each genus was identified from wound and skin swabs. The number shown in brackets is the total number of species identified in that genus. When a genus could not be identified to the species level it was reported as Spp. Spp was counted as 1 species.

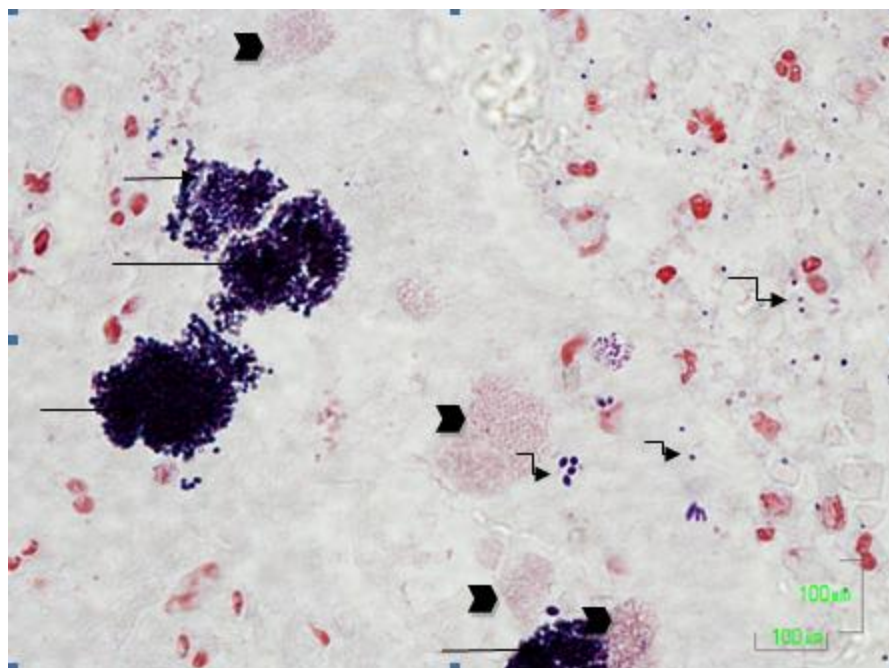


Figure 1. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing evidence of Gram negative (arrow heads) and Gram positive (long arrows) and individual colonies (elbow arrows). The epidermal edge of the wound is to the left of the image. (x1000 magnification).

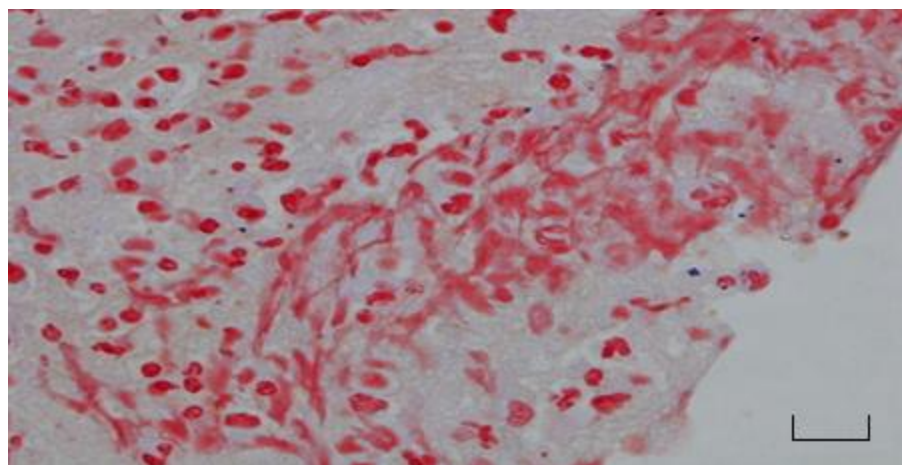


Figure 2. Gram stained debrided tissue taken from a chronic, non healing equine lower limb wound, showing an area of uninfected tissue. The epidermal edge of the wound is visible on the bottom right of the image. (x1000 magnification) (Bar +100 μ m).

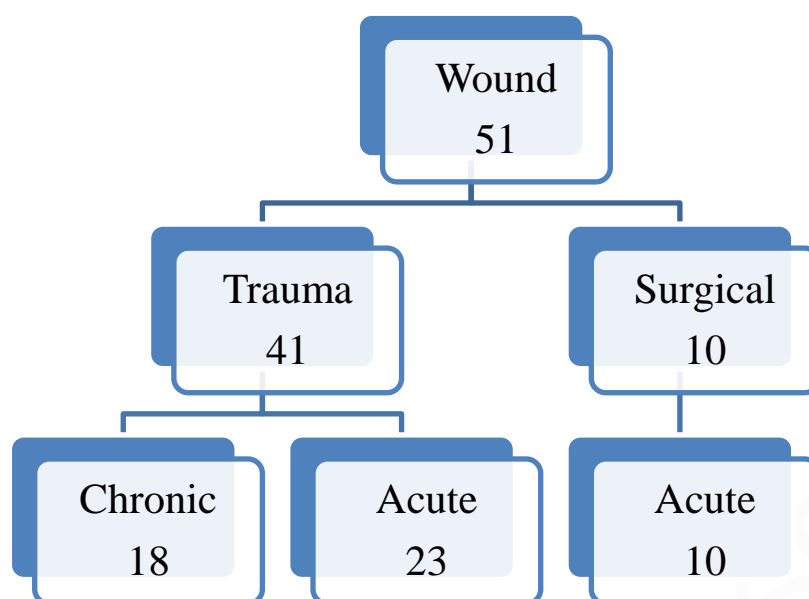


Figure 3. Hierarchical flow diagram showing the number of each wound type included in the study. Control swabs were also taken from the uninjured skin of all the 51 cases.

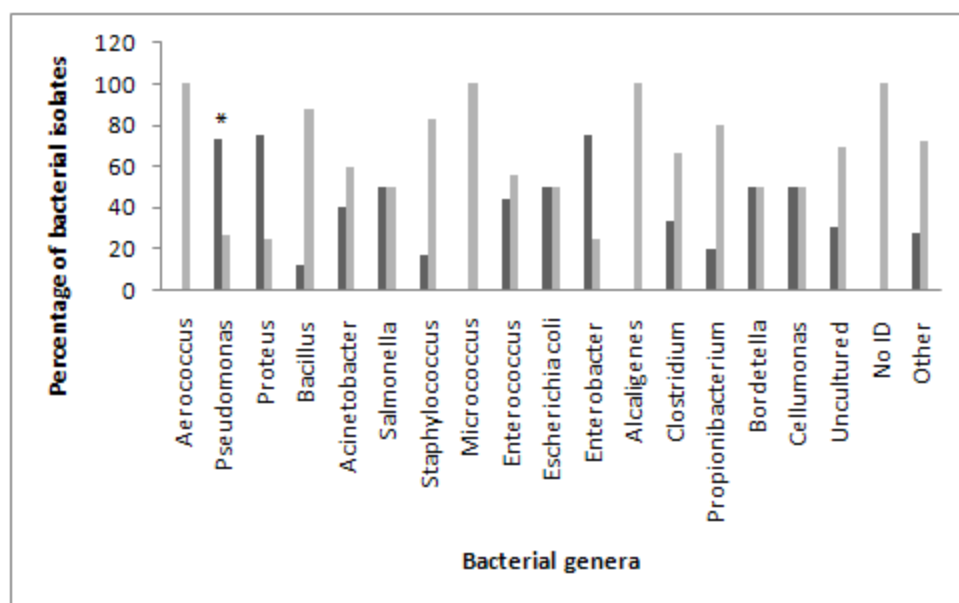


Figure 4. Percentage representation of bacterial genera isolated from chronic wounds (black bar) and acute wounds (grey bar) (n=51). * - significant difference between chronic and acute wounds $P < 0.05$.

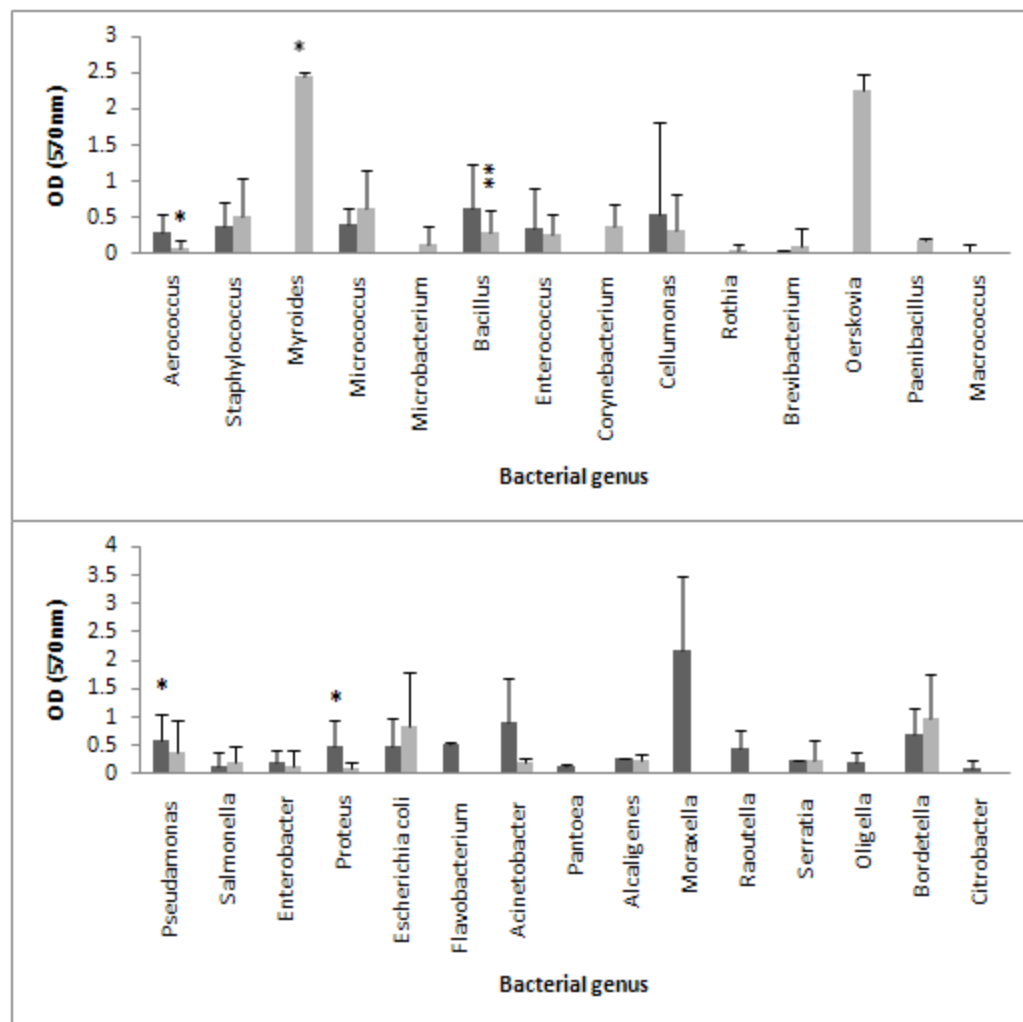


Figure 5. A comparison between the OD of wound (Black bars) and Skin (Grey bars) isolates.

The upper graph shows Gram positive isolates and the lower graph shows Gram negative

isolates. * show significant differences between wound and skin OD values * - $P < 0.05$, ** -

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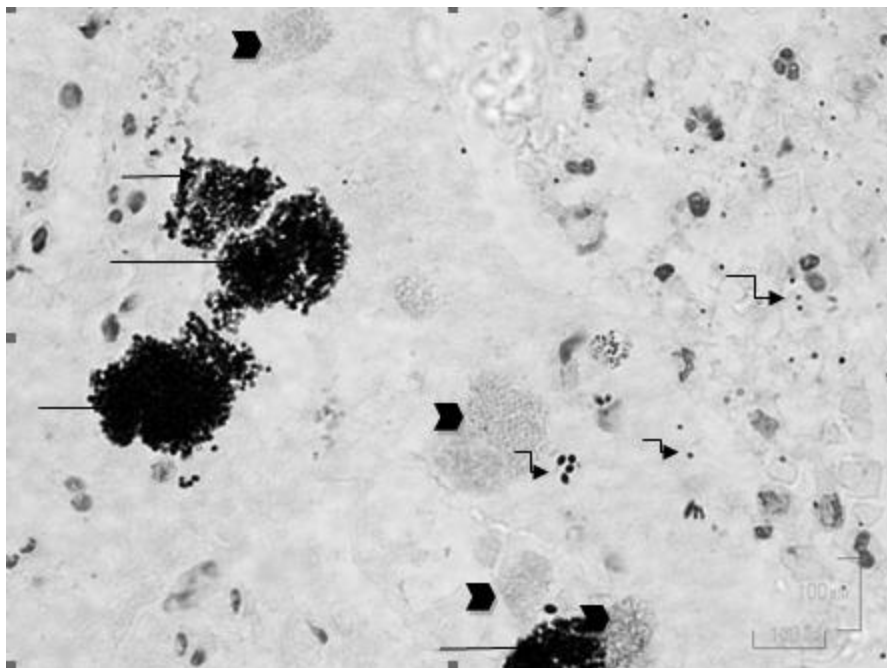


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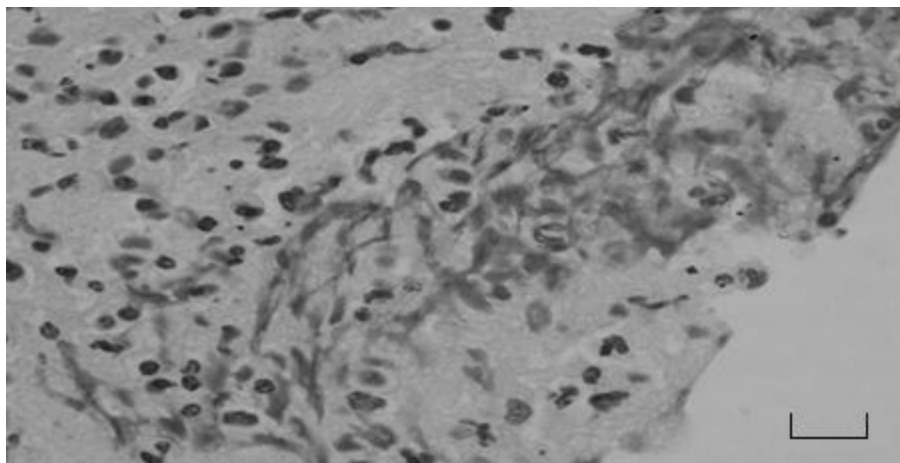


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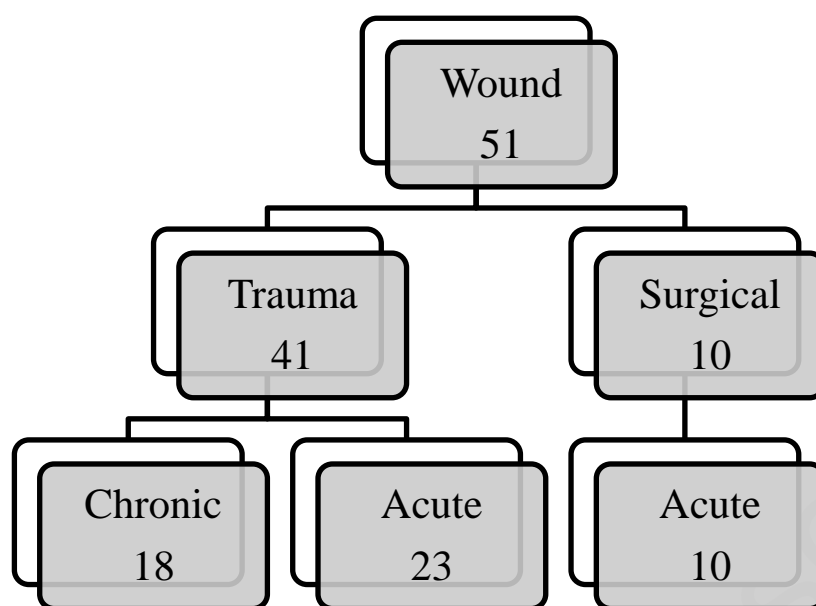


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