The importance of anaerobic bacteria in non-healing wounds

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ABSTRACT

Chronic wounds, with their polymicrobial nature, put a significant burden on health budgets worldwide. Bacterial presence in the wound bed is associated with poor healing. The role of anaerobic bacteria in wound healing impairment has not been definitively established, mainly due to lack of reporting resulting from culture-based limitations. Advanced molecular methods are more reliable and the presence of anaerobic bacteria can be detected.

We have analysed 207 wound swab samples from 38 non-healing wound patients. Samples were collected at the Queensland University of Technology (QUT) wound clinic (Brisbane) over a period of 12 weeks. Next Generation Sequencing (NGS) technology was used for determining bacterial diversity and abundance in these wound samples. We found multiple types of bacteria residing on the non-healing wound bed. Bacterial diversity results are discussed in this paper with a focus on the importance of anaerobic bacteria. Overall, different patients' wounds harbour diverse bacterial populations. Similarly, the presence of anaerobic bacteria in the wound bed was also detected. Molecular methods are reliable and useful, particularly for organisms requiring special conditions for growth such as anaerobes. It is clinically important to determine the major microbes present in the wound bed and their relationship with wound chronicity.

Keywords: Chronic wounds, wound healing, anaerobes, bacterial diversity, Gram-positive anaerobic cocci.

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INTRODUCTION

A wound is formed due to damage to the skin and when the normal protective function of the skin is lost[1]. Bacteria have been reported to affect the healing process by invading the wound surface, damaging the tissue and prolonging the inflammatory response[2-5]. A wound which remains for more than six weeks[6] or which does not progress to healing in four weeks[7] is classified as a chronic wound. Bioburden is a broader term which includes total bacterial load and diversity as well as any specific virulent or resistant organism. Wound healing has been reported to be affected by bioburden[8].

Various types of bacteria have been reported to be present in the wound bed. *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus (*MRSA), is the most common bacterial species involved in skin infections[1]. Gjodsbol and colleagues have studied 50 patients who had chronic venous leg ulcers. Their results show that these wounds mostly harbour a polymicrobial flora, as indicated by the presence of more than one bacterial species in 94.4% of the samples[9]. James and colleagues reported the presence of *Staphylococcus* sp., *Enterococcus* sp., *Pseudomonas* sp., *Proteus* sp., *Citrobacter* sp. and *Enterobacter* sp. using culture-based methods, while molecular analysis detected additional species[10]. The most common bacteria reported in another study are; *Staphylococcus* sp., *Pseudomonas* sp., *Peptoniphilus* sp., *Enterobacter* sp., *Stenotrophomonas* sp., *Finegoldia* sp. and *Serratia* sp.[11]. Likewise, *Streptococcus* sp., *Porphyromonas* sp., *Anaerococcus* sp., *Prevotella* sp., *Stenotrophomonas* sp., *Finegoldia* sp., and *Serratia* sp. have been reported in other studies as well[12,13].

Anaerobic bacteria have been reported to be involved in wound chronicity and biofilm production[2,14]. The presence of high percentages of anaerobic bacteria has been reported in pressure ulcers[15]. Similarly, anaerobic bacteria have been identified as a major contributor to wound bioburden[16].

Gram-positive anaerobic cocci (GPAC) are an important group of anaerobic bacteria and are associated with 25–30% of clinical infections[17]. Anaerobic bacteria need absence or limited availability of oxygen. They find a perfect environment in deeper layers of biofilm as oxygen diffusion is blocked by biofilm layers[18]. Currently, the most clinically important genera of GPAC are *Anaerococcus* sp., *Finegoldia* sp., *Parvimonas* sp., *Peptoniphilus* sp. and *Peptostreptococcus* sp.[17].

In general, GPAC infections are mostly controlled by beta-lactamase inhibitors, carbapenems, cephalosporins and chloramphenicol. In addition, variable levels of resistance have developed against antibiotics such as penicillins, clindamycin and metronidazole[19]. This variability in GPAC resistance and other anaerobic bacteria highlights the need to identify which anaerobes are mainly present in the wound bed and their impact on wound healing, so that wound infections can be promptly treated with appropriate antibiotics after identifying the relevant species/strains.

It is important clinically to determine which microbes are associated with chronic wounds and to detect their virulence determinants. There is a need to further increase our knowledge and understanding of the bacterial role in acute and chronic wounds[14]. Currently, there is no 'gold standard' for determining the correlation between bacterial load/bioburden and wound chronicity. It is, therefore, very important to develop more robust and accurate methods, such as molecular methods, to quantify bacterial load and diversity in chronic wounds. Once these methods have been developed and validated, wound treatment plans can be modified to improve healing outcomes[20]. This study aimed to identify bacterial diversity in non-healing wounds using advanced molecular techniques with its main focus on anaerobes.

METHODS

Samples: Wound swab samples were collected at the Queensland University of Technology (QUT) wound clinic, with ethics approval for human research (QUT project approval number 1000001255). Swabs were collected using the Z-technique and were stored at –80°C until further use. Wound nursing clinicians, specialised in wound care, at the QUT wound clinic, were responsible for wound swab sample collection. A set protocol for collection of Z-swabs was followed by all clinicians involved in collection of the samples.

Wound swabs were collected from patients undergoing chronic wound treatment (QUT wound clinic) over a period of 12 weeks in 2011. Patients whose wounds did not heal by week 24 from their initial presentation at the wound clinic, were selected for this study and were categorised as "non-healers" while "healers" were excluded from the study. In total, 207 swabs from 38 non-healing wounds were used for this study. Out of these 38 patients, 19 had mixed ulcers, seven had arterial ulcers, seven had venous ulcers, two had pressure ulcers, two patients had undergone amputation surgery and one patient's wound aetiology was unclear. These wounds were located at different areas of the lower extremities and all patients were receiving standard wound care at the clinic, including silver, hydro-fibre, hydrogel and zinc paste dressings. Prior to swab collection, wounds were washed with water. It was acknowledged that the use of antimicrobial dressings is likely to influence the microbial flora.

DNA extraction and Next Generation Sequencing (NGS): DNA extraction from swab samples was done following the Price *et al.*[21] protocol with additional physical and enzymatic lysis steps. PCR amplification was performed using fusion primers, which were designed from the universal 16S rRNA (prokaryotic small subunit ribosomal RNA). Samples were subjected to NGS using the Ion Torrent PGM platform. Sequencing data was integrated using the Mothur software program (http://www.mothur.org/) and was further analysed using the Calypso software program. These experiments were performed at the Central Analytical Research Facility at QUT.

RESULTS AND DISCUSSION:

The results show varying trends of bacterial diversity among different patients (n=38). Overall, the bacterial diversity changes with or without a dominant genus or genera; however, there were patients whose wounds were dominated by particular genera from the earlier stages of sampling and remained prevalent throughout the sampling course. There were multiple types of bacteria detected in our patients. In total more than 50 genera have been detected and the dominant genera present in different patients were *Staphylococcus* sp., *Bacteroides* sp., *Anaerococus* sp., *Peptoniphilus* sp., *Porphyromonas* sp., *Fusobacterium* sp., *Prevotella* sp. and *Finegoldia* sp. Thus both facultative and strictly anaerobic bacterial genera have been detected in this study. This is consistent with research findings in other studies. Additionally the polymicrobial nature of chronic wounds is also validated by these results.

This paper focuses on the importance of anaerobes only. The overall results of bacterial diversity and abundance from all 38 non-healing patients are part of a separate investigation. Likewise, the importance of biofilm in wound healing has been reported and discussed in recent literature. Anaerobes are also involved in biofilm production. Similarly, staphylococci and *Pseudomonas* species are also biofilm producers. Our research group has submitted an article reporting the presence and absence of common biofilm controlling genes, which includes a discussion about the role

of biofilm in wound chronicity. The following figures show results of bacterial diversity in the form of bar charts and bubble plots using the Calypso software for patient #4001 and patient #4059 as representative samples. These two patients are presented as an example.

In patient #4001, multiple genera were present with variations over the course of the study while in patient #4059 there was dominance of one genus *(Staphylococcus* sp.) throughout the study period. This variation in type and dominance was observed for several patients, with the majority of patients presenting with changing bacterial flora over 12 weeks of sample collection.

Figures 2a and 2b show results of bacterial diversity in the form of bubble plots, using the Calypso software, for patient #4001 and patient #4059. It is clear from these figures that patient #4001 has variation in bacterial diversity at different weeks with *Staphylococcus* sp. and *Anaerococcus* sp. the dominant bacterial genera present. On the other hand, patient #4059 clearly shows *Staphylococcus* sp. dominance throughout the course of the study. The scale under the bubble plot represents the percentage of each genus.

Culture-based techniques have been widely used to study wound bioburden and were regarded as the gold standard method for this purpose but molecular methods have produced more promising results[17,22]. Culturing is a slow and selective process that misses the majority of organisms including anaerobes, which are very important from the wound healing point of view[11,23,24]. Molecular diagnostic techniques provide a more diverse picture of the microbial content of wounds and/or biofilms[11]. Frank *et al*. proposed that it is worth combining culturing with molecular methods to achieve the best results[22].

Advanced techniques, such as NGS, are able to reveal further details regarding the microbial flora of clinical samples, including that of chronic wounds[11]. Han and colleagues have compared culture-based methods and NGS, and they have reported the presence of three main bacterial species (on average) using culture-based methods while pyrosequencing

Figure 1a

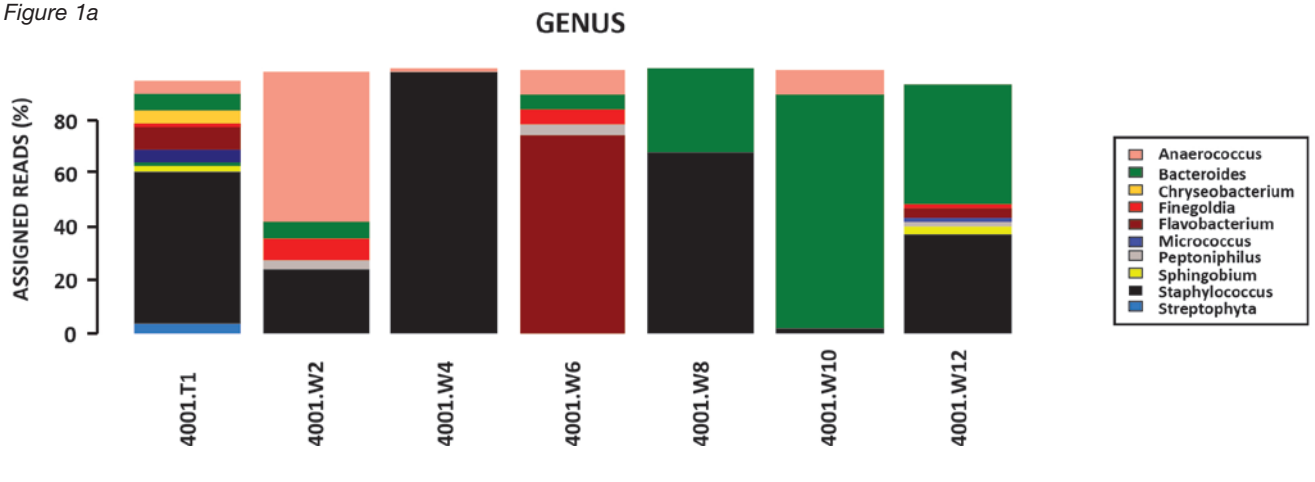
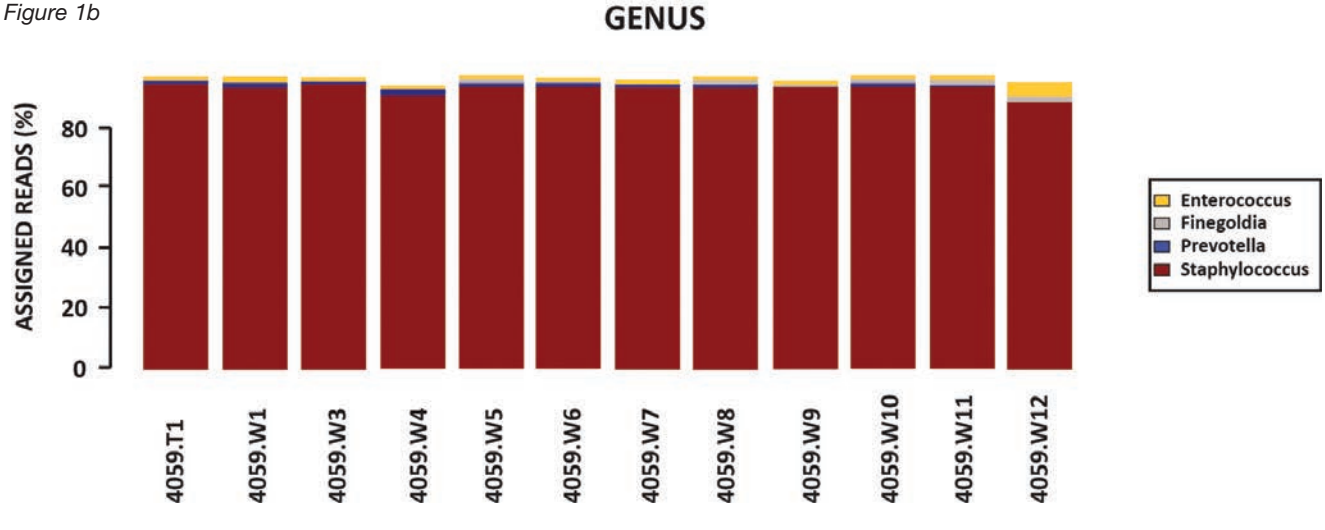


Figure 1b



on average detected 17 different genera. Most of the genera detected by NGS were anaerobic bacteria such as *Anaerococcus* sp., *Finegoldia* sp. and *Peptinophilus* sp.[16].

Dowd and colleagues[10] used pyrosequencing, shotgun Sanger sequencing and denaturing gradient gel electrophoresis for the investigation of bacterial diversity in chronic wounds and Gardner and colleagues[12] used 16S rRNA sequencing for bacterial diversity studies[13,15]. The development of molecular methods such as PCR, multiplex PCR, 16S rRNA sequencing and NGS has improved the detection and identification of numerous organisms, including anaerobic bacteria[17]. As mentioned previously, we also detected the presence of more than 50 genera in total in all of our patients (n=38)

Figure 2a

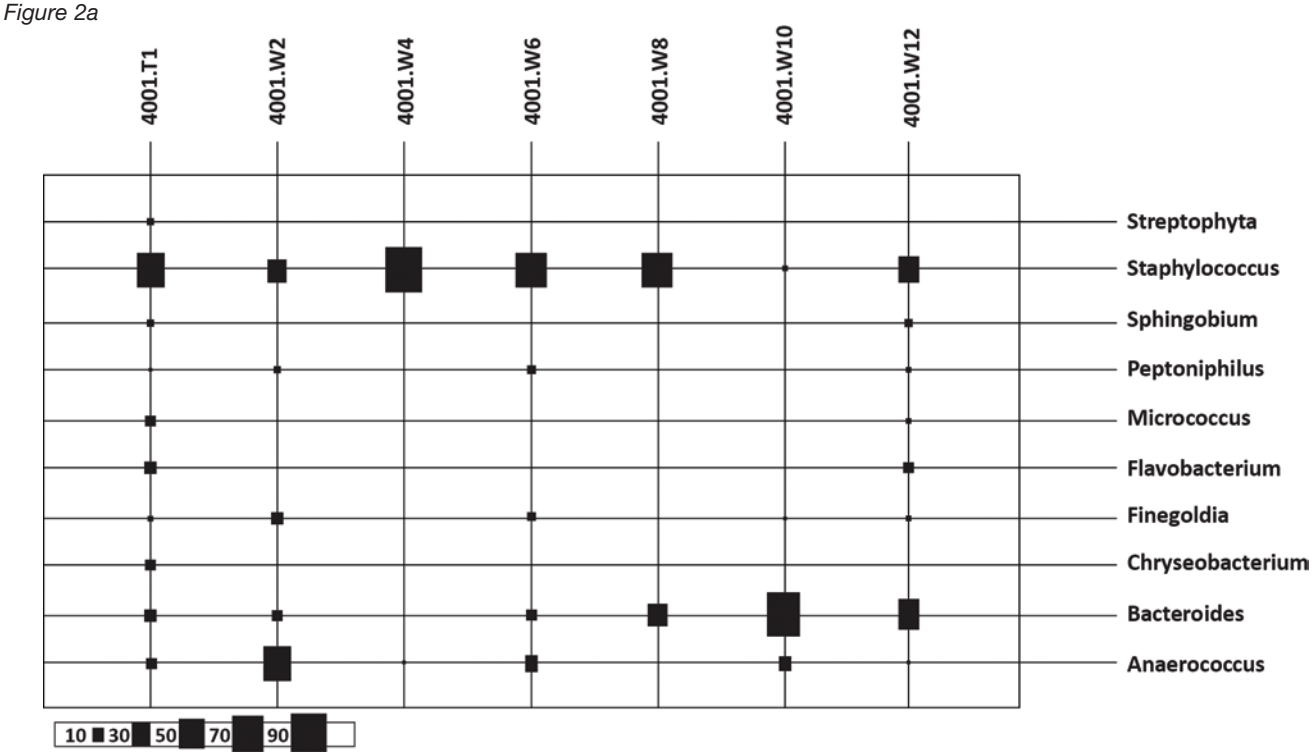
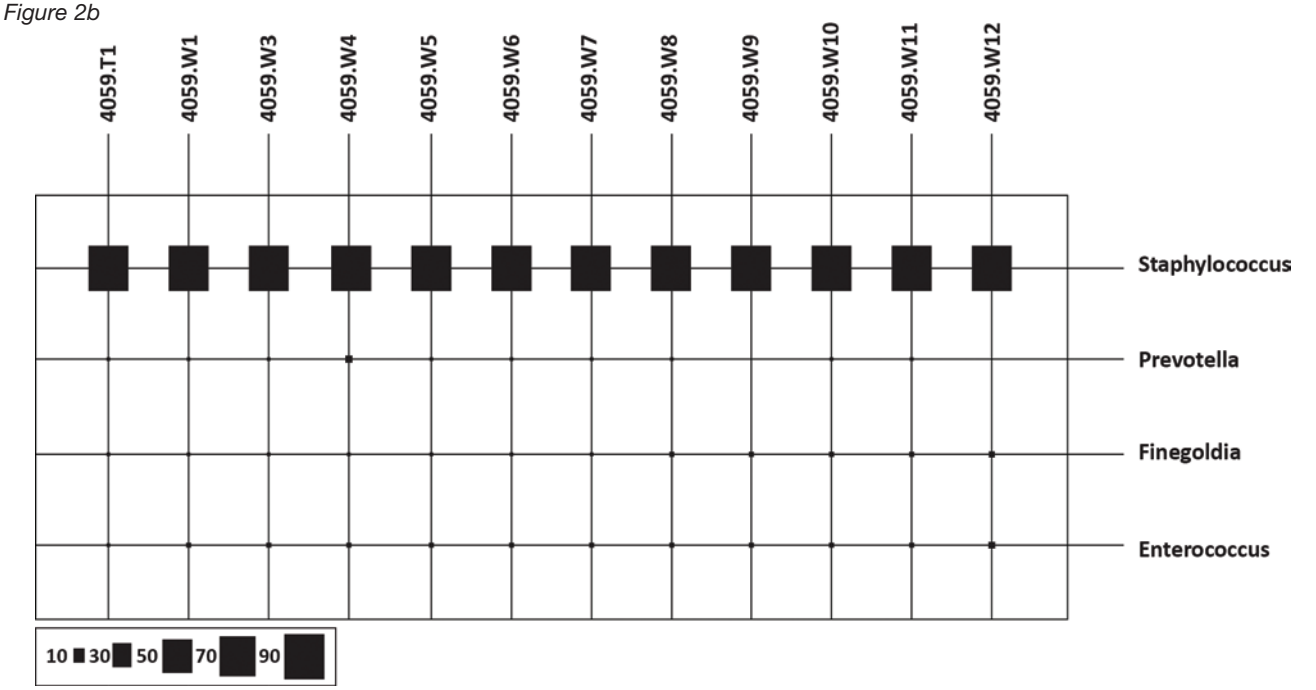


Figure 2b



with dominance of *Staphylococcus* sp., *Bacteroides* sp., *Anaerococus* sp., *Peptoniphilus* sp., *Prevotella* sp. and *Finegoldia* sp.

Anaerobic bacteria are usually cultured using blood or fastidious anaerobic agar and incubation periods vary from 48 hours up to 7 days[17]. Morphology, Gram-staining and sensitivity to antibiotics such as metronidazole are used to identify anaerobes in many laboratories[19]. Some GPAC species have been reported to have resistance to metronidazole, thus these organisms would be ignored if metronidazole sensitivity is a criterion for identification[19]. We are reporting the presence of anaerobic bacteria in our non-healing wound patient samples and suggesting their association with wound chronicity. The exact impact of anaerobes on wound healing as well as virulent determinants related to wounds requires further investigation. Wound management and treatment will improve by revealing details regarding wound microbial flora[17].

The clinical significance of GPAC has been underestimated for years, mainly due to two factors. Firstly, culture-based techniques usually fail to report anaerobic bacteria due to their slow growth and specific growth requirements in the laboratory. Secondly, they are usually present in infections involving multiple bacteria, thus other known pathogens have been given more clinical importance and the presence of anaerobic bacteria has been overlooked[17]. Samples for identification of anaerobic bacteria require appropriate collection, transportation and strict anaerobic culture conditions[17]. Culture-based methods have limitations, therefore molecular methods should be developed[25,26]. Advanced molecular techniques have increased GPAC detection and established their involvement in clinical infections. Furthermore, molecular methods have detected many new species in this group resulting in changes in taxonomy and nomenclature[17]. The availability and cost related to molecular methods is an important issue. However, we recommend the development of a standardised protocol for the evaluation of non-healing chronic wounds.

CLINICAL RELEVANCE OF FINDINGS

Based on the experimental results as well as the literature review, we are proposing the following:

1. Molecular techniques should be preferred over cultural methods to obtain a better picture of bacterial diversity in the wound bed. This is particularly important for the identification of bacteria that require special transport and culture conditions such as anaerobic bacteria.
2. Anaerobic bacterial infections need to be treated appropriately as they have been reported to delay healing and are involved in biofilm formation[2,14]. Furthermore, resistance to antibiotics has been reported in anaerobic bacteria[27,28].
3. We recommend regular monitoring of wound bed flora, particularly in non-healing wounds as our results show new bacterial types can grow over the course of treatment and replace the originally detected bacterial type(s). This means there might be a need to further add antibiotics or change them. Regular evaluation and change in the treatment plan has been found to reduce the total cost of treatment[29]. For this purpose, a clinical evaluation of wounds, involving wound treating physicians and staff is required. Clinical signs of infection such as redness and heat cannot be totally relied on as these are usually not very prominent in chronic wounds due to several factors, including diseases such as diabetes mellitus, and the use of drugs which suppress pain and inflammation[30].

AUTHOR DISCLOSURE AND GHOSTWRITING

No conflicts of interest exist. The content of this article was expressly written by the authors listed. No ghostwriters were used to write this article.

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