Infection update

Wounds International clinical updates present recent developments in the field of leg ulcers, pressure ulcers, skin integrity and diabetic foot, including the latest from associations, clinicians and industry. If you use an innovative technique in your practice that you would like us to feature in future issues, please email the editor at: *scalne@woundsinternational.com*

Methods for microbial identification in chronic wounds

It is now well established that bacteria can use two different strategies for producing infection[1]. The most widely recognised is when bacteria propagate as single motile cells to invade host tissue. The bacteria is usually a single species and will upregulate virulence factors to kill the host cells. The bacteria will then secrete bacterial-derived proteases to break down host tissue, which is used as nutrition for continued propagation. This planktonic strategy is most consistent with what is known clinically as an acute infection.

Wound biofilm

A less recognised, almost paracystic strategy, is when bacteria attach to host cells and/or tissue to produce an infection. The act of attachment causes upregulation of biofilm genes, which produce a protective matrix and organise the bacteria into a polymicrobial community[2]. To protect itself, the community, through a variety of secretory systems, 'infects' the host cells with small effector proteins which render the host cells senescent[3] (where the host cell ceases to function appropriately).

Using a variety of molecular mechanisms, the wound biofilm blocks host cell apoptosis (programmed cell death),[4-7] shedding[8], migration[9] and manufacturing,[10] among other functions. This produces a stable attachment to the host environment while also preventing the host from healing. These are the unique properties of a biofilm infection most consistent with the chronic infections seen clinically.

Biofilm is present in chronic wounds[11], yet much work remains in order to establish the exact contribution a biofilm makes to the non-healing of an individual wound. It must be remembered that an individual bacterium possesses the genetic material necessary to either pursue a biofilm or planktonic mode of growth. This means that the bacteria present in any chronic wound can change from one mode of growth to the other.

There is no question that a biofilm, with its colony defences, is the more difficult phenotype to diagnose and treat. Therefore, the focus of diagnostic and treatment methods should be on biofilm, since any planktonic bacteria will also be adequately dealt with by the same process.

Accuracy of routine cultures

Bacteria seem to play an important role in the non-healing of wounds. Therefore, it is important to choose diagnostic methods that can fully identify both planktonic (usually single species) and biofilm (usually polymicrobial) phenotypes present in the wound.

Cultures have been shown to be wholly inadequate in identifying bacteria in biofilm phenotypes and any polymicrobial infections[12]. Cultures select against the large numbers of species present in polymicrobial infections by growing only a few species favoured by the conditions. Therefore, cultures are not quantitative and the biochemical identification methods are inaccurate.

Recent findings

A recent study which compared clinical cultures with molecular diagnostics uncovered several interesting findings. The clinical cultures were processed by a high-volume clinical lab, which routinely evaluated wound samples. The molecular methods included real-time polymerase chain reaction (PCR) — the use of a specific primer to identify an organism — combined with pyrosequencing — a

Figure 1: Sequencing instruments in a dedicated analysis room.

Figure 2: Extracting microbial DNA for analysis from wound samples.

method used to determine the DNA code (adenine, thymine, cytosine and guanine) for a specific gene.

In the 168 wound samples evaluated, molecular technology identified 338 unique genera, whereas culture methods revealed only 17 different genera. Cultures failed to grow the vast majority of micro-organisms present in the wound. It should also be noted that 41 samples out of the 168 wounds cultured identified bacteria that molecular methods could not confirm.

Given the high level of accuracy of sequencing and PCR it seems most likely that culture reported erroneous results. The important generalisation from the study is that culture methods are not adequate in identifying bacteria in a polymicrobial infection.[12]

A second study (recently submitted for publication by the author's group) looked at clinical culture versus DNA identification for 51 chronic wounds. Molecular methods identified 17.7 genera per sample whereas clinical cultures grew only 1.8 genera per sample with a maximum of five in a single sample. The major finding was that up to 59% of the aerobic bacterial load was not identified by culture. Another important finding was that clinical cultures reported micro-organisms that were less than 1% of the wound bioburden 17% of the time.

Far from avoiding the reporting of minor populations, cultures amplify minor populations a significant portion of the time. Also, a crossover analysis using molecular methods to re-identify the micro-organisms present on the subculture plate shows that a culture identification misidentified 18% of the micro-organisms that grew.

Given the findings of these two studies, cultures seem inadequate for evaluating the bacteria present in wounds. As reported above, cultures sometimes (about 25% of the time) report bacteria that are not shown as present using molecular methods and misidentify bacteria 18% of the time. Thus, molecular methods are felt to be more reliable.

However, since there is currently no widely accepted 'gold standard' for microbial identification in medicine, individual clinicians have to decide which results — PCR, sequencing or culture — they feel are more reliable.

Cultures have a significant selection bias, amplifying bacteria that grow easily, even if they are at low levels in the sample initially. But the fatal flaw for cultures in diagnosing wound bioburden, and one that no methodological improvements will overcome, is the inability of the culture to fully identify the diversity present in an individual wound.

Even though most molecular methods for identifying bacteria are in their pioneering stages, they are more sensitive and specific than cultures. PCR methods can identify bacteria with high accuracy down to a few bacterial cells in just two to three hours. Yet, PCR methods only identify the bacteria for which primers have been developed. To identify the vast number of bacteria that have not had primers developed, sequencing methods must be used[13]. Sequencing methods can be used to determine the exact order of the DNA bases (adenine, thymine, cytosine, guanine) of a very specific region (ie 16S rDNA gene), to determine a 'bacterial fingerprint' for each bacteria present in the infection.

This method allows for comprehensive identification of all micro-organisms along with quantitation, or how many of the micro-organisms are present. The downside of sequencing is that it takes three to four days to complete. However, by combining PCR and sequencing methods, the clinician can receive rapid information as to important pathogens — Methicillin-resistant *Staphylococcus aureus* (MRSA); Vancomycin-resistant *Enterococcus* (VRE) and multi-drug resistant *Pseudomonas aeruginosa* (MDRPA) — in real time, and then comprehensive data can be returned in three to four days.

A second strategy utilising molecular methods is to combine different technologies. This has been performed using the rapid power of PCR to amplify the 16S fingerprint region in a clinical sample. The amplified 16S rDNA gene can then be examined by mass spectroscopy instead of sequencing to identify a very broad range of micro-organisms. This method provides results that are similar to sequencing, but, as with PCR, in only two to three hours[14]. Currently, however, this technology lacks the breadth and DNA certainty of sequencing.

This hybrid technology also lacks the ability to evaluate highly diverse infections. This highlights the difficulty of developing a single molecular diagnostic test that meets all the needs of the clinician.

However, new molecular technologies, such as the hybrid technology Plex-ID (Abbott), which combines PCR and mass spectroscopy, are emerging which may provide all the important microbial as well as host information from a chronic infection, such as a non-healing wound. Clinically, these new molecular methods mean a more rapid, accurate and comprehensive diagnosis of the wound microbiota.

Currently, there is scientific method that can reject any micro-organism as being unimportant in a clinical infection. Therefore, it is necessary to identify and quantify all microbes present in a chronic wound. It has been shown in large cohort studies that employing molecular methods to direct the use of commercially available wound care products (topical iodine, silver, methylene blue etc), along with antibiotics,

improved healing at six months[15]. Similarly, the use of molecular methods to guide the use of personalised gels improved healing further at six months [16]. The gels contain high quantities of antibiotics and anti-biofilm agents that specifically target any bacteria present. It may be that, by comprehensively suppressing wound biofilm, chronic wounds will heal more effectively.

Conclusion

With the emergence of clinically meaningful diagnostic tests, such as PCR and sequencing, wound care can move away from trial and error to the more standard medical model of diagnosis followed by treatment. However, today's molecular methods are in the pioneering phase and still face barriers in terms of being widely accepted. This is due, in part, to the high cost of the tests and the need for highly trained personnel, but is mainly because these methods yield a high-quantity data, which is currently unfamiliar.

However, new technologies are rapidly emerging, which will provide more accurate, faster, cheaper and far more comprehensive identification of all microbes in chronic wounds. Molecular methods are certain, therefore, to become the preferred way of identifying microbial presence in chronic wounds.

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Innovations in wound infection management

Infection in chronic wounds still remains a major problem in wound management since it sometimes leads to lethal complications, such as osteomyelitis, cellulitis, bacteraemia and sepsis. Once wound infection occurs, it can easily develop into these conditions, thus early detection and diagnosis is essential. The difficulty in diagnosing wound infection is often due to the absence of the typical inflammatory symptoms, including pain, redness, swelling, and heat, which indicate critical colonisation *[Fig 1]*. Since critically colonised wounds cannot be defined by clinical signs, clinicians sometimes fail to treat the wound in its early stage of infection. The authors proposed a new way of identifying these conditions by focusing on gene expression of bacteria and host. The aim is to introduce their strategy and discuss future issues regarding wound infection control.

The relationship between bacterial virulence and host immune response is generally conceptualised as a state of balance. If the bacterial virulence surpasses the host immunity, the wound will not heal and will lead to infection or critical colonisation. In contrast, if the host immunity overwhelms the bacterial virulence, the wound will heal without any excessive inflammatory signs, described as colonisation or contamination.

Diagnosing infected or critically colonised wound is usually based on bacterial count and clinical features.

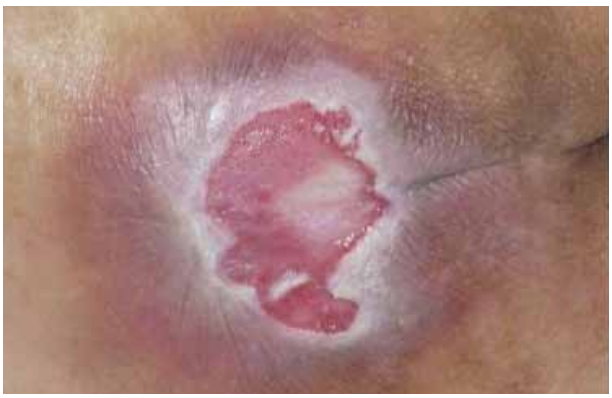

Figure 1. This image shows a critically colonised pressure ulcer. Although the wound lacks the typical inflammatory signs and symptoms, wound healing did not progress despite optimal treatment. After the application of antiseptics, the wound started to heal.

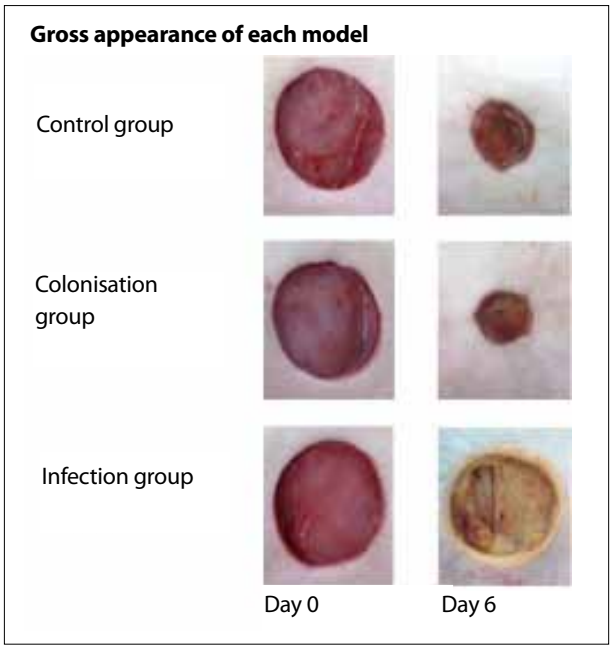

Figure 2. Unlike the control and colonisation group, the infection group shows obvious signs of infection, such as slough and necrotic tissue spreading to the periwound area, as well as differences in the level of the wound bed and edges. One representative result from the three groups is shown.

The authors claim that the 'gold standard' for diagnosing wound infection is counting the colony-forming units (CFUs). By using this method, wound infection is defined in international pressure ulcer guidelines as 'a bacterial bioburden of >105 CFU/g of tissue and/or the presence of beta-haemolytic streptococci'[1].

However, it is difficult to diagnose wound infection based solely on bacterial numbers because the virulence factor differs among bacterial species and interspecies. Bacterial synergy means that a mixed infection with two or more species of bacteria results in worse outcomes than single bacteria-induced infection. Furthermore, it is already well-known that more than 99% of bacteria cannot be cultured *in vitro* and are termed as 'viable but non-culturable'. This problem also hinders clinicians from performing bacterial counts to diagnose wound infection.

As a result of these problems, it is recommended by the authors that clinicians assess the inflammatory signs and symptoms when diagnosing infection, even though this will not detect the critical colonisation.

Method for detecting wound infection and critical colonisation

Delays in detecting wound infection or critical colonisation result in further complications. The authors, therefore, attempted to establish a new strategy for solving this clinical challenge. Since they already knew that wound infection is the result of an imbalance in bacterial virulence and host immunity, they postulated that analysing this balance would be a marker for detecting wound infection.

However, because both the bacterial count and the clinical signs and symptoms focus on either aspects of this balance, they are unsuitable for clinical use. Therefore, from this point, the authors focused on gene expression analysis because the biological responses to wound infection can be directly assessed at the messenger RNA (ribonucleic acid) (mRNA) level. Furthermore, the authors adopted the reverse transcription-polymerase chain reaction on the centrifugal precipitation of wound fluids (termed 'wound fluid RT-PCR').

Since wound fluid is easily collected and contains both bacterial and host cells, the authors thought this method may be promising when assessing the balance of bacteria and host relationship. Moreover, it is noteworthy that wound fluid is easily collected and RT-PCR is highly sensitive, thus clinicians can analyse the gene expression level from a small amount of samples, meaning this method would be noninvasive.

To confirm its usefulness in diagnosing wound infection, the authors performed animal experiments. Different doses of bacteria were administered to the wound to make three wound states — control, colonisation, and infection. Using rat models featuring a wound that had not been inoculated (control), a wound with low dose bacterial load (colonisation), and a wound with high-dose bacterial load (infection), the authors extracted the mRNA from the centrifugal precipitation of the wound fluid leaking from the wound beds *[Fig 2]*.

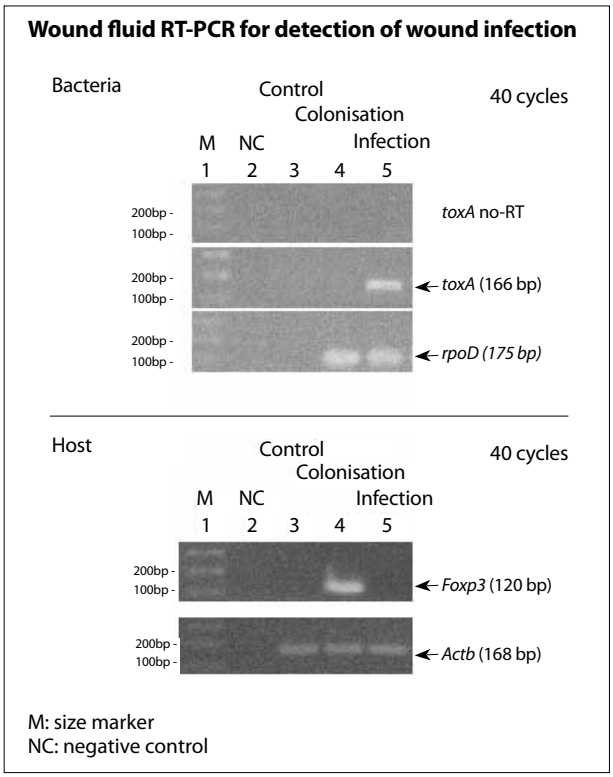

Figure 3. While the expression of rpoD (a gene specific to **Pseudomonas aeruginosa***) indicates the presence of bacteria in colonised and infected wounds, only toxA was detected in infected wounds. Of the host maker genes, only Foxp3 was detected in colonised wounds. The combination of expression in bacterial and host genes can offer a useful clue to detecting the wound infection.*

By screening many candidate host genes related to immunocompetence, wound healing, and apoptosis, as well as bacterial genes related to exotoxin and biofilm formation, the authors discovered the combination of genes for discriminating the infected wound from a colonised wound, which was not possible by counting the bacterial number from biopsy samples.

From the host genes, the expression of *Foxp3*, encoding regulatory T-cell (Treg)-specific forkhead box transcription factor Foxp3, was only expressed in the colonisation group. On the other hand, from the bacterial genes, the expression of *toxA*, encoding virulence factor exotoxin A, which is regulated by quorum sensing system, was only detected in the infection group[3] *[Fig 3]*. By exploring these specific marker genes, the authors shed light on the establishment of appropriate diagnosis of wound infection and they believe the clinical application of this concept could be put into practise in the near future.

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Expert Commentary

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Of the thousands of microorganisms in existence, there are only a limited number that colonise the human host. Some can cause disease (pathogens) and the remainder (non-pathogens) cannot because they do not have the mechanisms to do so. This is not always consistent and we now know that a microorganism can lose and acquire pathogenic (virulence) genes. We know these virulence genes can be switched on and off by environmental factors and more recently, we have come to accept that microorganisms do not grow as single species (as we see in the laboratory), but as a community or biofilm in the host. In addition, we know that there can be an interaction of a pathogen and a non-pathogen and the combined effect on the host can be different to the effect of the two individual microorganisms.

Current diagnostic microbiology is still based on the postulates introduced by Robert Koch (1843-1910) over a century ago and rely on the isolation of a pathogen from an infected site. Following isolation, a potential pathogen is identified and reported along with an antimicrobial susceptibility to assist in the treatment of the patient. These cultural methods have been the mainstay of traditional diagnostic microbiology and have helped our understanding and ability to control many bacterial diseases, such as typhoid, meningitis, tuberculosis and cholera. Development of non-culture techniques (molecular and immunological) and a fuller understanding of exactly how microorganisms cause disease has raised a large number of questions and has also led to numerous research projects in all aspects of infectious disease. The introduction of molecular techniques has allowed the study of the epidemiology and potential treatment of a wide range of microorganisms without ever having to culture them.

The techniques outlined in the two articles featured here have been applied successfully to difficulties with accurate diagnosis of wound infection and critical colonisation. The data captured in the first article by Wolcott and colleagues show that there is a greater diversity of bacterial species present in chronic wounds within a biofilm than previously reported using traditional methods. The relevance of these findings suggest that it is not always the presence of an individual microorganism (or pathogen) that causes problems with wound healing, but the interactive effect of the polymicrobial community contained in a biofilm. Whether the comprehensive identification and diversity of all the different species of microorganism in a wound will make a significant impact on diagnosis and treatment has yet to be determined.

The quantitative detection of extracellular biological molecules (biomarkers) released into the wound by the microorganism and/or in combination with host molecules, highlighted by Nakagami and colleagues, shows great promise as an alternative diagnostic culture tool. If detection of certain biomarkers in a wound is shown to differentiate between microbial colonisation and infection then this would help the practitioner make urgent treatment choices when necessary. Molecular techniques are slowly moving into the hospital pathology laboratory, but are still too costly and time consuming for diagnosis of many infections, and traditional methods continue to be used. Where the causative microorganism is a communicable threat or a life-threatening disease (eg tuberculosis, meningitis) molecular techniques have been introduced , but often at large university hospitals or the Health Protection Agency Reference Centre in the UK, rather than in routine hospital laboratories. Their future introduction into hospital laboratories is dependent upon cost, usability and applicability to treatment.

The application of these non-cultural techniques to chronic wounds and wound care in the research setting has helped with the understanding of why some wounds do not heal, especially in a patient where they should. The understanding that a biofilm exists on many surfaces in chronic wounds has shown that accurate sampling can be difficult and full identification of microorganisms within a wound by conventional techniques is almost impossible. The reporting of potential pathogens with antibiotic sensitivities will continue to be the normal practice of routine diagnostic laboratories until other techniques have been proven to have more patient benefit and are cost effective.

Further development of non-cultural methods that detect biological markers can certainly help the clinician move towards more understanding and accurate diagnosis of wound infection or wound colonisation, especially if these assays can be used near the patient at the point of care. This would give the clinician more confidence to administer antibiotics and/or effect a change of treatment in the form of debridement, topical antisepsis or perhaps radical surgery.