Bacterial diversity in surgical site infections: not just aerobic cocci any more

• **Objective:** To evaluate the microbial diversity in chronic surgical site infections (SSIs).
• **Method:** Bacterial populations in 23 chronic SSIs were identified using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), which is an universal bacterial identification method. These results were then validated using quantitative polymerase chain reaction (qPCR).
• **Results:** bTEFAP identified two previously uncharacterised *Bacteroidales* in all of the SSIs and showed that it was the predominant population in the majority of these chronic wounds. Other bacteria identified included *Corynebacterium* spp., *Peptoniphilus* spp., *Staphylococcus* spp., *Staphylococcus aureus, Serratia marcescens, Prevotella* spp. *and Pseudomonas aeruginosa*. Rarefaction analysis of the data indicated that, on average, six genera occurred in any given SSI, suggesting that such infections are multispecies. On average, over 60% of the bacteria evaluated in the SSIs were anaerobic bacilli. The previous literature indicates that aerobic cocci predominate in such wounds.
• **Conclusion:** This modern molecular survey indicates that our previous understanding of which bacteria cause SSIs may be faulty. The high prevalence of anaerobic bacilli and the overwhelming predominance of two previously uncharacterised *Bacteroidales* suggest that such bacteria may be a leading contributor to such infections. Further research on the identification and treatment of such bacteria are warranted.
• **Declaration of interest:** Scott E. Dowd is director of a clinical molecular diagnostic company.

surgical site infection; biofilm; *Staohylococcus aureus;* bTEFAP; qPCR

It was generally thought that Gram-positive aerobic cocci, particularly *Staphylococci*, are the primary cause of surgical site infections (SSIs).[1,2] Indeed, the isolates most commonly identified in SSIs using culture-based methods are *Staphylococcus aureus*, coagulase-negative staphylococcus, *Enterococcus* spp. and *Escherichia coli*.[1-3]

However, this does not take account of the fact that the biofilm phenotype, with its multispecies communities, is the natural state of existence for most types of bacterium, and that over 99% of bacteria identified in every environment are organised in biofilm communities.[4] Indeed, it is now becoming accepted that not only are biofilms the prevalent cause of chronic wound infection,[5] but also that culture methods cannot identify biofilms.[5-7]

SSIs have many of the characteristics of chronic wounds specifically and of chronic infections in general.[5,6] Their management therefore requires an understanding of biofilm phenotype bacteria.[8]

Few studies have used modern molecular methods to evaluate the microbial diversity in SSIs. Previous studies have relied on clinical and laboratory culture methods to evaluate which organisms can be isolated in pure culture from an SSI. As *Staphylococcus, Streptococcus, Escherichia and Pseudomonas* spp. grow easily in clinical and laboratory culture media and are easily isolated, it is not surprising that they are the microorganisms most frequently associated with SSIs. However, throughout the literature it is stated that, on average, fewer than 5% of all bacteria can be easily grown in laboratory culture media. Therefore, 95% of all bacteria that might be associated with or causative factors for SSIs are never isolated and so have never been identified. This limits our ability to treat such infections. Molecular techniques have an advantage over culture methods as they do not rely on growing limited species of bacteria in the laboratory, but instead are able to identify all microorganisms contained within a sample on the basis of their genetic material.[9-12]

Examples of molecular techniques that pose an alternative to traditional bacteriological analysis are bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) and quantitative polymerase chain reaction (qPCR). bTEFAP is a molecular technology that uses genetic information to identify all bacteria contained within a wound sample. qPCR is another mo lecular detection technique that enables us to detect the specific genetic signatures of bacteria and provide relative or even absolute quantification of that genetic material.

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Table 1. Patient demographics and wound characteristics

Sample code	Sex	Age	Diabetes	Location	Duration (months)
1	Female	56	No	Left lateral ankle	2
1b	Female	57	No	Left lateral ankle	6
2	Female	61	Yes	Left biceps	3
3	Female	46	No	Right ankle	3
4	Male	54	No	Right forearm	2
5	Male	87	No	Right hip	6
5b	Male	88	No	Right hip	8
6	Female	80	Yes	Right below-knee amputation	6
7	Female	73	No	Right elbow	1
8	Male	66	No	Left plantar first metatarsal	4
9	Male	40	No	Abdomen	12
10	Male	40	No	Right shoulder	5
11	Male	73	No	Abdomen	18
11b	Male	72	No	Abdomen	5
12	Female	48	No	Right hernia	6
13	Female	49	No	Mid back	2
14	Male	72	No	Right knee	3
14b	Male	72	No	Right knee	4
15	Male	74	No	Abdomen	22
16	Male	43	No	Left heel	1.25
17	Male	21	No	S/P Harrington Rod back	2
18	Male	68	No	Chest	12
19	Female	41	No	Abdominal	10

This study used bTEFAP to identify all microorganisms found in 23 wounds with chronic SSIs. It then validated these results by using a second independent measure — qPCR. The combined use of these techniques enabled us to evaluate the diversity and predominance of all microorganisms identified in each sample. We anticipate, therefore, that this study will shed light on the diversity of SSI-related microbial communities and demonstrate that a reliance on clinical culture has limited our knowledge of this.

Method

Debridement samples from 23 separate SSIs were collected from patients at the Southwest Regional Wound Care Center (Lubbock, Texas) in accordance with Western Institutional Review Board protocol number 20062347. The debridement samples were collected with sterile tools after cleansing the wound using sterile techniques. All samples were frozen in sterile collection tubes at -80°C until DNA extraction was performed. Table 1 provides a summary of these 23 SSIs. The inclusion criterion was wounds classified as SSIs of at least one month's duration that had been referred to Southwest Regional Wound Care Clinic.

DNA extraction

After thawing, portions of the debridement samples (mean 200mg ± 100) were recovered using sterile forceps. The samples were placed in 2ml sterile micro centrifuge tubes, centrifuged at 14,000 revolutions per minute (rpm) for 30 seconds and resuspended in 500µl RLT buffer (Qiagen, CA, US) with β-mercaptoethanol.

A sterile 5mm steel bead and 500µl 0.1mm sterile glass beads (Scientific Industries, NY, USA) were added to achieve complete bacterial lysis in a Qiagen TissueLyser, run at 30Hz for five minutes.

Samples were centrifuged briefly and 100µl 100% ethanol was added to a 100µl aliquot of the sample supernatant. This mixture was then added to a DNA spin column, and DNA recovery protocols were followed as instructed in the QIAamp DNA Mini Kit (Qiagen).

DNA was eluted from the column with 30µl water, and samples were diluted accordingly to a final concentration of 20ng/µl for use with all qPCR reactions. DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

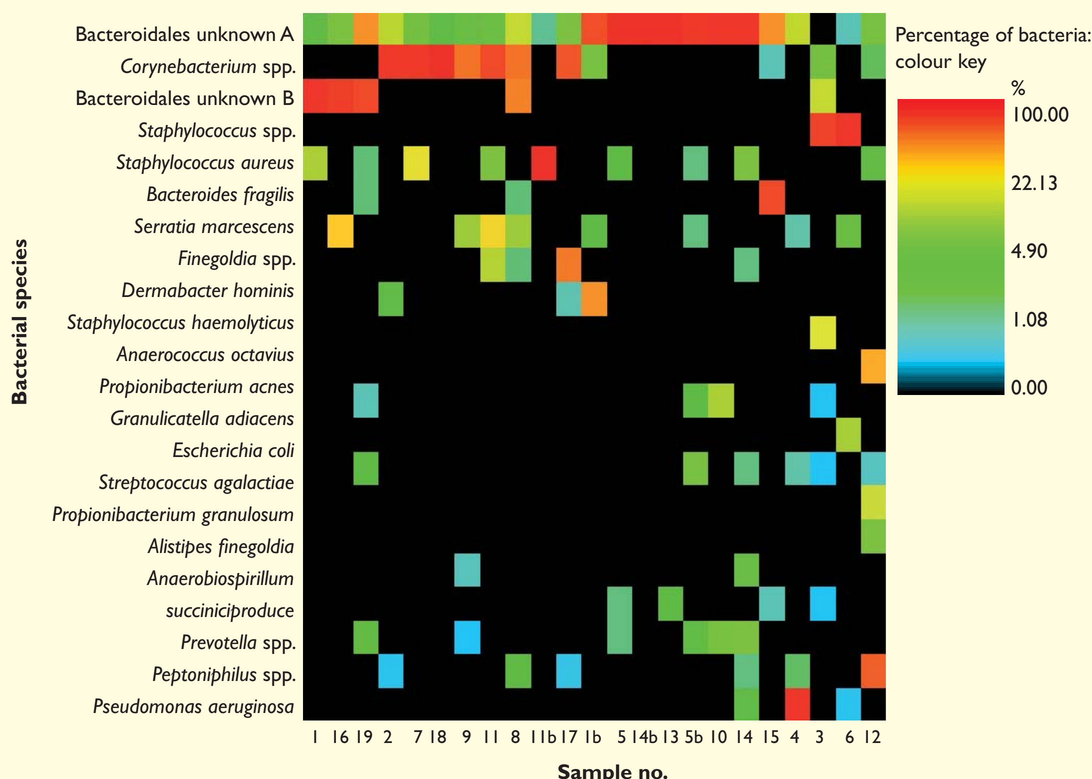
bTEFAP

bTEFAP was performed as described previously.[9-11]

- **bTEFAP sequence processing pipeline** Once the DNA sequence data for each sample are generated, the goal is to take the raw genetic information and manipulate it to determine the bacteria present within each sample and their relative percentages. Processing removes poor-quality or noisy genetic data, organises it and then uses a computer algorithm to identify the microorganisms from which the data were derived.

Custom software written in C# within a Microsoft .NET development environment was used for all post-sequencing processing. In summary, quality-trimmed sequences were derived directly from FLX sequencing run output files. Tags (artificially created genetic signatures used to identify which sample or specimen the sequence was derived from) were extracted from the FASTA files (which contained all

Fig 1. Diversity and distribution of species among the SSIs



of the sequence information) into individual sample-specific files, based on the tag sequence. Tags that did not have 100% homology to the sample designation were not considered. Sequences less than 150bp after quality trimming were not considered.

The resulting sequence information was parsed among the 23 samples, averaging at least 1,000 sequence reads per sample. These were then depleted of chimeric (contaminated) sequences and evaluated using Basic Local Alignment Search Tool (BLAST) against a custom database derived from GenBank (http://ncbi.nlm.nih.gov).

A post-processing algorithm generated a summary of the genetic search-engine data for each sample. Bacteria classified at the species level had a sequence identity (divergence) greater than 96.5%; at the genus level, it was between 94% and 96.5%; and at the closest family level, it was less than these values. No sequences were analysed with less than 84% similarity from known sequences.

Following best-hit processing, a secondary post-processing algorithm was used to compile data from each sample and determine the relative predicted ratios of each organism within each sample.

qPCR

The diagnostic panel described previously was used.[13] Each sample was screened to determine the relative percentage of *S. aureus, Pseudomonas aeruginosa, Serratia marcescens and E. coli*, and validated by comparing this with the results obtained using bTEFAP.

Two unknown *Bacteriodales* were identified using the above techniques. One of these, unknown *Bacteriodales* A, was further characterised and validated using SYBR green qPCR. Using full-length DNA sequences obtained from the sample, quantitative primers were designed using a custom computer program. To validate and confirm the percentage of the unknown *Bacteroidales* A, the results were compared with an universal eubacterial quantitative SYBR green specific primer set. This allowed us to detect this unknown bacterium and determine that the original sequencing information is reproducible.

Statistics

Statistics were performed using the comparative functions and multivariate hierarchical clustering methods of NCSS 2007 (Kaysville, UT, USA). Rarefaction analysis was performed as described previously.[11]

Table 2. Summary of the primary genera identified in the SSIs

Name	No. of samples	Mean %	SD	Maximum %	Gram stain*	Oxygen tolerance†	Morphology (shape)
Bacteroides	23	45.4	42.1	100	-	Anaerobic	Rod
Staphylococcus	13	29.9	41.1	99.4	+	Facultative anaerobe	Cocci
Prevotella	11	2.9	5.8	20	-	Anaerobic	Rod
Corynebacterium	11	41.5	38.2	98.8	+	Aerobic	Rod
Peptoniphilus	6	9.7	21.9	54.5	+	Anaerobic	Cocci
Escherichia	6	1.0	1.4	3.8	-	Facultative anaerobe	Rod
Serratia	6	4.8	6.4	17.2	-	Facultative anaerobe	Rod
Finegoldia	5	9.4	16.4	38.2	+	Anaerobic	Cocci
Propionibacterium	5	2.7	3.1	7.6	+	Anaerobic	Rod
Anaerobiospirillum	4	0.5	0.4	1.1	-	Anaerobic	Sprial
Pseudomonas	3	31.6	48.1	87.0	-	Aerobic	Rod
Streptococcus	3	4.2	5.4	10.5	+	Facultative anaerobe	Cocci
Dermabacter	3	10.7	17.4	30.8	+	Aerobic	Rod
Anaerococcus	2	12.9	16.6	24.7	+	Anaerobic	Cocci

No. of samples = the no. of samples in which the genera were identified
Mean % = the average percentage of that genera in each of the samples
SD = standard deviation of these percentages
Maximum % = the maximum percentage of the genera in each sample

* + Gram-positive; - Gram-negative
† Anaerobic bacteria are unable to propagate in laboratory media in the presence of oxygen; facultative anaerobes can grow both in the presence of oxygen and without it; aerobic bacteria can grow in the presence of oxygen

Results

The bTEFAP results are shown in Fig 1, which provides an overview of the diversity data and depicts the distribution of bacterial species among the SSIs. The colours provide a relative indication of the prevalence (percentage) of each species within a given SSI (sample x-axis). Red indicates a high percentage, while black indicates absence of a given genus/species. The scale on the left provides the key to the heat map colours. Fig 1 indicates that:

- *Bacteroidales* unknown A isolate occurred in all but one of the SSIs, and was the predominant population in seven samples
- *Bacteroidales* unknown B was predominant in three samples
- One of the two unknown *Bacteroidales* (A and B) occurred in all of the SSIs and was the primary population in 11 of the 23 SSIs
- *Corynebacterium* spp. were the predominant populations in seven of the samples and occurred in 12
- *Staphylococcus* spp. (probably representing a new species of *Staphlococcus*) was predominant in two of the samples
- *S. aureus* occurred in nine samples but was predominant in only one.

Other bacteria that occurred frequently were *S. marcescens, E. coli, Anaerobiospirillum succiniciproduce, Prevotella and Peptoniphilus* spp. *P. aeruginosa* occurred in three samples but was predominant in only one.

Table 2 provides a summary of the primary genera identified in these SSIs. The two species most frequently identified were *Bacteroides* and *Staphylococcus* (primarily *S. aureus*). The predominance of anaerobes such as *Peptoniphilus, Finegoldia* and *Anaerococcus* was also notable. This is supported in Fig 2, which shows that anaerobic bacilli predominated in the SSI samples.

Further summary of Fig 2 illustrates that the anaerobes were the predominant populations in well over half the samples, with facultative aerobes occurring as predominant populations in only nine.

The predominance of bacilli and anaerobes contradicts previous reports that aerobic cocci are the primary contributor to SSI.[9,11] The most ubiquitous

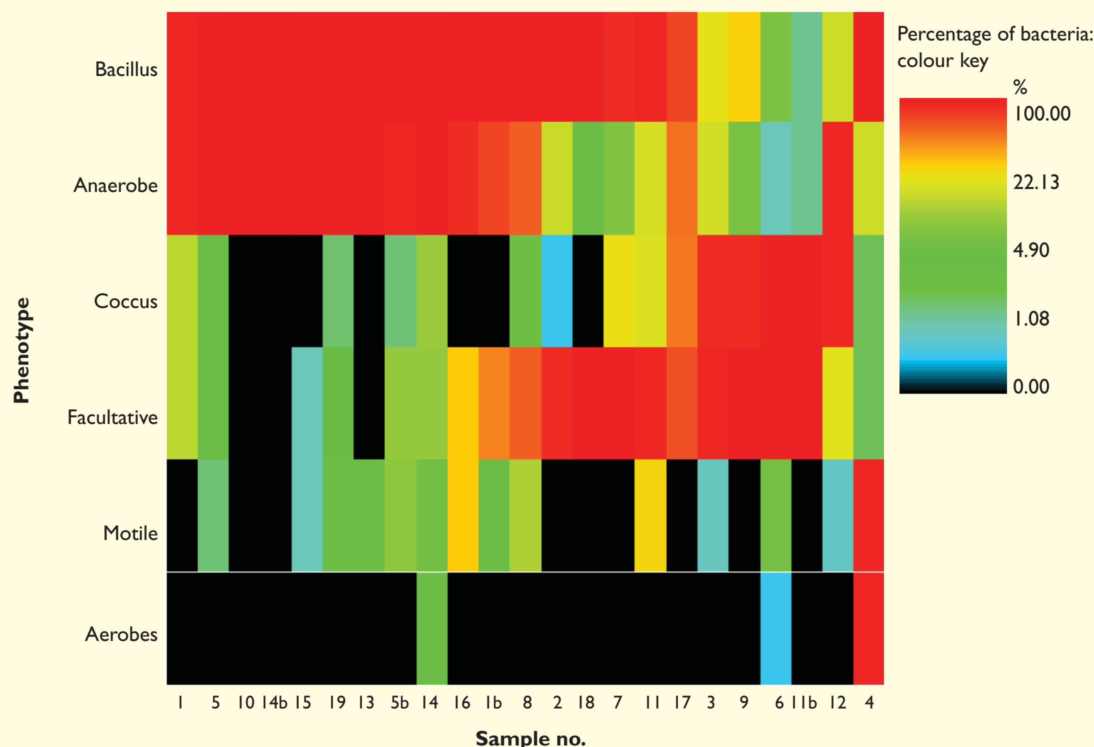


Fig 2. Distribution and contribution of major phenotypic bacterial characteristics from surgical site infections

organisms were two previously uncharacterised bacteroidales, which occurred in all but two of the SSIs, suggesting that these unknown anaerobes may be major contributors to SSI infections. The novel *Bacteroidales* organism is closely related to *Bacteroides* spp. The samples were all unrelated; the sampling dates ranged over a 12-month period and there was no correlation between the sampling date and the occurrence of these organisms (data not shown).

Results of the qPCR correlated highly with the bTEFAP results (85%, $p<0.01$), indicating that the bTEFAP results were accurate.

Finally, we evaluated the diversity of bacteria among the wounds. Rarefaction analysis, which was performed as described previously,[11] showed an average of 10 bacterial species in each sample, calculated at 97% similarity, and an average of six genera, calculated at 95% sequence similarity. This indicates that SSIs are typically multispecies infections, with an average of six genera in each wound and an average of 10 bacterial species.

Discussion

We hypothesised that either a single major 'culturable' pathogen, such as *S. aureus*, would be associated with all such wounds or that no single pathogen is associated with them. The latter hypothesis suggests that a mixed-species biofilm community, as opposed to a lone pathogen, causes the chronic infection observed in chronic SSIs. The results indicate that a diverse population of bacteria was usually present in these samples.

Most of the bacteria identified were anaerobic. These anaerobic rods may not respond to typical therapies, which are targeted at facultative aerobic cocci and are usually based on aerobic culture-based diagnostics. Our results support other studies that used molecular techniques and consistently found *Bacteroides* in chronic wounds.[11,14-16]

Corynebacterium spp. occurred in 11 of the samples and was the predominant genus in seven. Similar results were obtained in an earlier study evaluating bacterial diversity in DFUs using the same broad survey approach as here.[9] The most ubiquitous genus identified was a previously uncharacterised species of *Corynebacterium*, which was found in 30 out of the 40 ulcers. *Bacteroides* and *Peptoniphilus* were also highly prevalent and were present in 25 and 25 samples respectively.[9]

Corynebacterium is an underappreciated pathogen. Other studies have associated it with diabetic foot osteomyelitis, as reviewed previously.[17] In the study noted in this review, traditional culture methods identified the fastidious (hard to grow in the laboratory) *Corynebacterium*, which is commonly considered a contaminant, as a pathogen in DFU-associated

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osteomyelitis. However, the easily cultured *S. aureus* is commonly considered the primary pathogen in ulcers in general.

We suggest that culture techniques overestimate the importance of organisms that are easily cultured and can underestimate that of fastidious organisms, such as anaerobes and *Corynebacterium*, and certainly this new *Bacteroidales*.[18] Indeed, we have been unable to culture and isolate this new *Bacteroidales* in the laboratory using the most advanced anaerobe culture methods.

Anaerobes such as this newly discovered *Bacteroidales* are beginning to be recognised as major populations in chronic wound biofilms.[12,19-23] The importance of anaerobes such as *Peptostreptococcus, Prevotella, Finegoldia* and *Peptoniphilus* has been reported.[19,24-28] This is supported by the present study, which found that these genera represent a significant portion of SSI microbiota.

Even though wounds are typically exposed to air,[25] anaerobes may be the most prevalent physiological type in a given wound or an individual wound type. Bowler et al.[25] evaluated infected VLUs using cultural isolation techniques that included special considerations for the propagation of anaerobes. They reported that anaerobes represented 49% of the total microbial composition. Dowd et al.,[9] using a pyrosequencing approach, reported that 30% of the DNA sequences from pooled chronic DFUs were anaerobes and later confirmed that, in individual DFUs, anaerobes were also a predominant part of the biofilm ecology.[9]

Laboratory studies have shown[29,30] that obligate anaerobes may cope with the toxic effects of oxygen by interacting with aerobic or facultative bacteria populations in a symbiotic manner as part of a process known as coaggregation. Aerobic species may consume oxygen and create localised niches, allowing the obligate anaerobes to gain an advantage when in close proximity to their oxygen-reducing neighbours.

Rasmussen et al. have also shown that oxygen only penetrates microns into the surface of biofilms, suggesting that internal regions of the bacterial communities may support only anaerobes and facultative anaerobes.[31]

It has been proposed that sequencing the 16S gene of clinical, laboratory-cultivated bacteria, as is the case with bTEFAP, has advantages over traditional biochemical identification methods.[32] We propose that not only is bTEFAP more effective in identifying cultivated microbes, but such assays can also be universal methods of pathogen diagnosis. Indeed, our findings highlight the shortcoming of relying on culture methods to identify important bacterial populations within clinical samples.

Infections that are predominantly caused by bacteria in the planktonic phenotype tend to be acute, with a significant host response that is characterised by the classic signs and symptoms including erythema, pain, swelling and heat. The hallmark of acute infections is that they are susceptible to antibiotics and resolve in 10–14 days.[33]

Planktonic-phenotype bacteria explain much of this behaviour. Planktonic and motile bacteria upregulate virulence factors, bacterial proteases and other secreted agents to lyse tissues, on which it then feeds.[34] The perceived pattern of acute planktonic infection is one of predation: if the host does not adequately respond or there is no outside intervention, the host dies.

Many SSIs, however, occur after discharge and show a slow, undulating course that is considerably different to an acute infection. Often the entire incision will dehisce, but there is no degradation of tissue surrounding the wound. The damage is most often confined to the surface of the surgical incision. Biofilms are more successful on surfaces, especially interfacing surfaces.[35] Also, even though culture methods demonstrate at least some of the bacterial species present in biofilms, antibiotics are unsuccessful in eradicating most of these infections.[36] The presence of a biofilm on the surface of the surgical wound may explain why we stand by helplessly while our planktonic tools and strategies fail to prevent the wound from dehiscing.

Biofilm management of SSIs is based on multiple concurrent strategies that specifically target biofilm behaviour.[7] This includes opening any tunnelling and undermining by removing sutures or opening skin to expose the surface-associated bacteria. This robs the biofilm of a second surface to organise around, and creates access for other strategies. The biofilm can be deprived of its nutritional source by immunosuppressants, but this blocks host-healing responses and should be considered a last resort in SSIs. Topical negative pressure may be substituted as it draws off exudate, potentially limiting the nutrient supply.

Frequent debridement of the wound surface forces the biofilm to constantly reconstitute itself and makes it more susceptible to antibiotics and selective biocides.[36,37] Using antibiotics at high doses (2–10 times above the minimum inhibitory concentration [MIC]) for 6–8 weeks[38] **(**improves biofilm suppression.[39]

As Fux[39] explicitly indicated, antibiotics alone will rarely be successful against biofilms and should only be used with other strategies. Selective biocides such as silver or cadexomer iodine will suppress biofilm phenotype bacteria up to one half log, but do not harm host healing responses.[40,41]

Biofilms are best managed through physical disruption. This principle has been proven in our baths, toilets and on our teeth. It has also been demonstrated in packaging, processing and pool maintenance. By frequently disrupting the biofilm

with brushes or by other physical means, the colony is degraded. Then, as the colony tries to reconstitute itself, treating agents such as biocides, antibiotics and even quorum-sensing inhibitors becomes more effective. This principle can be exploited in several ways. Consider a quick opening of the involved area of the wound, removing any dead and devitalised tissue frequently, and then physically or enzymatically managing the wound surface to suppress the re-accumulation of biofilm at weekly intervals.

Such physical disruption of the biofilm is rarely sufficient in itself. By adding other simultaneous strategies, such as selective biocides, antibiofilm agents and antibiotics, it is often possible to suppress the biofilm accumulation. When the biofilm is suppressed, host healing processes like angiogenesis, extracellular matrix formation and wound contraction become much more effective. It has been demonstrated that, by targeting biofilms, a higher percentage of chronic wounds heal, demonstrating that biofilm is an important barrier to healing.[7] Early intervention with aggressive, multiple, concurrent strategies that target surface-associated bacteria on the SSI may therefore improve outcomes.

This study is the first to use next-generation pathogen-detection methods to evaluate the diversity of bacteria in SSIs. Furthermore, it involved a broader range of patients than did most other studies that used molecular methods on these wounds. Still, only 23 samples were analysed from a single geographical region. Future work should look at a larger population from different geographical regions to gain a better understanding of the microbial populations associated with such infections.

Conclusion

We have used advanced, next-generation, molecular methods to evaluate the microbial ecology of SSIs. Previous literature, which has relied on outdated laboratory culture techniques, has stated that Gram-positive cocci, such as *S. aureus*, are dominant. In contrast, our results suggest that anaerobic rod-shaped bacteria predominate in biofilms. The inability to grow such bacteria using standard culture-based methods explains the historical inaccuracy of this information. Newer diagnostic methods, such as bTEFAP, may enable us to better target therapies to the pathogens in chronic wounds.

Finally, we have identified two previously uncharacterised *Bacteroidales* as ubiquitous and primary populations in most SSIs. Work is already underway to isolate and characterise these unknown *Bacteroidales* and define therapeutics for their control. This study may thus represent the initial description of a novel anaerobic pathogen associated with SSIs. ■

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