Microbiota is a primary cause of pathogenesis of chronic wounds

Objective: Diverse microorganisms present on the surface of chronic wounds have been established to constitute wound microbiota. The aims of this study were to quantify the viability of wound microbiota, classify dispersal of viable microbes from the wound, and determine if human wound microbiota can produce a chronic wound in an animal model.
Method: Wound microbiotas as units (multiple microbial species acting as one infectious agent) were obtained from well-defined human chronic wounds and seeded onto mouse surgical excision wounds to produce chronically infected wounds that closely resembled the chronic wounds observed in the original hosts.
Results: We found the wound microbiota harvested from 35 out of 43 (81%) patients could produce similar chronic wounds (producing slough and exudate) in a murine chronic wound model. The top 30 species present in patient wounds were identified in the mouse wounds by molecular sequencing. Koch's postulates could therefore be applied to establish wound microbiota as the cause of the original human chronic wound infections. Evidence-based medicine criteria such as Hill's criteria for causation can all be satisfied by what is currently known about wound microbiota.
Conclusion: This study demonstrates that wound microbiota actively disseminates from the chronic wound by forces and mechanisms intrinsic to the wound. Koch's postulates and Hill's criteria for causation together suggest chronic wound microbiota to be the main cause underlying the pathogenesis of chronic wounds.
Declaration of interest: RW has an equity interest in PathoGenius Labs. No funding was received for this study.

chronic wound • Koch's postulates • microbiota • pathogenicity • biofilm

Clinicians and scientists universally agree that all chronic wound surfaces contain microorganisms, collectively referred to as wound microbiota. Although experts concur that every chronic wound has certain microbiota (one or more species of microorganisms present and propagating within the wound), there is wide disagreement, and many varied concepts, on the role of microorganisms within a chronic wound.

The behaviour of wound microbiota is not completely understood; however, one can be certain that specific behaviours, including modes of growth, are highly influenced by microbial diversity, environmental, genetic factors and host factors. As presented in Table 1, there are many different concepts on the contribution of wound microbiota to the chronicity of wounds. Microorganisms can accumulate in vast numbers and in almost an infinite number of species combinations, suggesting the possibility of finding an extreme case that would demonstrate the validity of any of the concepts listed in Table 1.[1–14] Upon combining these concepts into a theory of the role of wound microbiota in the pathogenesis of chronic wounds, the null hypothesis would be that wound microbiota does not cause chronic wounds.

With classic methods and new evidence-based models, it is possible to gain reasonable certainty regarding the contribution of wound microbiota toward the pathogenesis of chronic wounds. The ability of microbes to propagate within a wound has been proven scientifically in multiple animal models by seeding lesions with bacteria and then demonstrating continued bacterial growth and survival in the chronic wound at later time points.[15–17] It is assumed that the microbes within the chronic wound disseminate into the environment in an effort at self-perpetuation. However, no study has specifically tested whether wound microbiota spreads in an active or passive fashion, i.e., whether the microorganisms actively (by intrinsic wound mechanisms and forces) leave the wound or if an external force is necessary to passively remove the microbiota from the wound. Dressing changes have been found to disperse microorganisms into the air and local environment.[18] Dressing studies also clearly show the formation of biofilm within dressings,[19] yet the spread of bacteria to the dressing might be via either mechanism. This is an important point in terms of its implications for managing dressings, environmental contamination, and to fully understand chronic wound microbiota. Most importantly, the US Food and Drug Admistration (FDA) has necessitated the clarification of whether the microbiota of a chronic wound seeds the environment actively, passively, or in both ways.

Of the two widely recognised modes of growth for microorganisms—planktonic and biofilm—it is highly likely that both phenotypes are dynamically present in wound microbiota. The degree to which one phenotype

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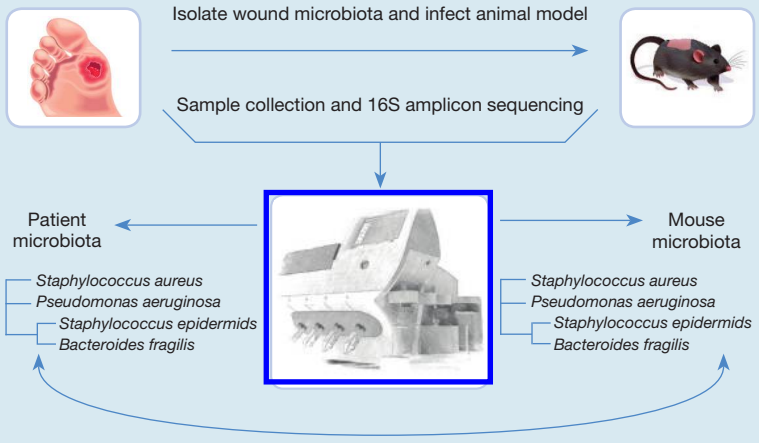
Table 1. Concepts of wound microbiota

Concepts of wound microbiota	Ref
A small number of microbes on the wound bed producing inflammation which improves wound healing	1–3
The microorganisms in chronic wounds (without primary signs of infection) are colonising the wound (propagating within the wound but not harming the host)	4,5
Multiple species of microorganisms are present on the surface of the wound yet just one species causes infection and the rest are contaminants	6,7
The wound microbiota is not producing immediate infection but is a reservoir for wound infection once host conditions are appropriate	8,9
The microbiota on the surface of the wound is well controlled by host defenses therefore it is usually the microorganisms that leave the wound bed and then propagate in dressings that release factors that do harm to the host	10–12
The wound microbiota forms biofilm producing a chronic infection, which uses radically different strategies for infection relative to planktonic phenotype	13,14

Fig 1. A modern recapitulation of Koch's postulates



dominates over the other may have considerable implications for an individual wound but may not play a significant role in determining if the microbiota is viable and capable of producing infection in a new host. Therefore, it is imperative that native wild-type wound microbiota containing both phenotypes be used to investigate the pathogenicity of wound microbiota.

If wound microbiota disperses and finds a new host environment that is permissive to its propagation, it might populate the new environment. That is, the wound microbiota would produce the same microbiological conditions as found in the original chronic wound. This is the basic premise of Koch's postulates: a specific microorganism is indeed the 'causative agent' of an infection if it can produce the same infection in an animal model and then be isolated from the experimental animal infection (Fig 1). While multiple weaknesses in Koch's postulates have been reported, the basic simplicity of the model continues to be scientifically compelling.[20]

Evidence-based medicine has embraced newer precepts, such as the Bradford Hill theory, which looks at multiple attributes of causation to establish the degree of correlation between a cause and effect.[21] This highly nuanced evidence-based approach accounts for vectors, synergistic causes, nonlinear causes, and several other factors occasionally encountered in biological systems. Many subtle points need to be addressed when trying to determine the presence of 'infection' and/or its 'cause.' These are as follows: Is a wound 'infected' if the bacteria simply use the host niche to propagate and do no harm? How much and what kind of 'harm' must the microbiota cause to constitute infection? For example, is recruitment of neutrophils into the new wound bed detrimental because it limits neutrophil activity for other important host needs or because excess neutrophils can produce tissue damage? Moreover, what if the microbiota is inducing inflammation past a point that has been shown to be beneficial for a cutaneous wound? Thus, terms such as 'cause' and 'infection' are often difficult to define.

This study represents an effort to document the viability, dispersal, and infection capability of wound microbiota. In other words, we aim to ascertain if microbiota can leave the wound actively and produce a new chronic wound in an animal model. A chronic wound is defined as a wound that demonstrates abnormal wound healing as evidenced by, but not limited to, delayed closure, exudate plus accumulation of slough. In the present study, microbiota affecting the host by abnormal wound healing with documented microbiota will be defined as infection.

Methods

Patients who participated in this study provided consent under a protocol that was approved by the Western Institutional Review Board (WIRB PRO NUM: 20062425). All elements of this study were considered to pose less than minimal risk to the patients, and each patient was fully informed and educated through the consenting process. All patient identifiers were removed from all study data, and only the clinical research coordinator securely retained the documentation linking an individual patient to study data.

Patients with highly exuding chronic wounds of any aetiology for more than a month were approached to participate in the study. After patients agreed and provided their consent, the study protocol was executed. Each patient was treated with biofilm-based wound care methods within the Southwest Regional Wound Care Center.[22]

Wound microbiota sampling

For the active exudate cohort, each patient was placed in a sitting or supine position, with the highly exudative wound mostly in a dependent position to the limb, allowing gravity to carry the wound exudate. Adaptic Touch (Systagenix, a division of Acelity, San Antonio, TX) 7.62 cm × 10.16 cm dressing was applied over the highly exudative wound. Next, a 47 mm diameter disc

of 2 mm pore size filter paper (Whatman, GE Healthcare Life Sciences, Pittsburgh, PA) was placed on the Adaptic Touch dressing. The study wound was not cleansed or prepared in any way before the application of the experimental material. After 5–10 minutes, depending on its saturation, the filter paper was removed and placed, with the wound-bed side of the filter paper up, on the base of a small petri dish. The petri dish was covered and stored at room temperature for a maximum of 2 hours before being transferred to the laboratory for analysis.

The study wound was then cleansed with normal saline as part of our usual standard of care. Next, the patient's wound was biopsied under local anaesthesia and subjected to sharp debridement using sterile curette, scissors, and/or scalpel to remove slough and devitalised tissue from the surface of the wound, which were then transferred to a sterile 2 ml tube. One tube per sample was stored for a maximum of 2 hours at room temperature before being taken to the laboratory for analysis.

Determination of bacterial cell viability and identification of microorganisms present in the slough were performed using propidium monoazide (PMA) staining combined with 16S amplicon sequencing, and by quantifying colony-forming units (CFUs) as described below. For quantifying CFUs, samples were cut into approximately 250 mm^3 pieces (average: 80 mg), transferred to a 2 ml tissue grinding tube with 2.4 mm metal beads and 1 ml sterile phosphate-buffered saline (PBS), homogenised (Precellys 24, Bertin Technologies Rockville, MD) at 5000 rpm for 60 seconds, serially diluted, plated on tryptone soy agar with sheep blood medium, and incubated at 37 °C aerobically for 24–48 hours. Filter paper samples were processed similarly. For CFU analysis, the filter paper was cut into approximately 2 cm × 2 cm sections, sonicated in 1 ml PBS to remove cells from the paper, serially diluted and plated on tryptone soy agar with sheep blood medium, and incubated at 37 °C aerobically for 24–48 hours. These same sonicated filter paper samples were used to infect mice.

Mouse model

As previously described,[23] 47 adult female Swiss Webster mice were anaesthetised, administered full-thickness, surgical excision wounds, and infected with approximately 125 mm^3 pieces (average: 30 mg) of intact human wound slough directly onto the wound bed, while 11 mice served as uninfected controls. We excluded four murine samples due to procedural or sample transport complications. Another group of 13 mice were wounded, and their wounds were inoculated with 100 µl of the filter paper homogenate solution described above. There were three mice used as controls, and their wounds were inoculated with 100 µl sterile PBS. Mouse wounds were imaged at the beginning and end of each mouse experiment with the SilhouetteStar camera (Aranz Medical, Christchurch, New Zealand) to monitor wound area and perimeter (data not shown) and visual purulence. After four days post inoculation (dpi), mice were euthanised, and wound tissue was excised and cut into equal pieces to test for viable microbial load by culturing, to determine total microbial viability by PMA staining, and to determine microbial population.

We determined that a four-day murine infection was appropriate, as this granted sufficient time for microbes from the human slough to colonise the new host and the murine wounds to visually develop slough on the murine wound bed. Yet it was not so long as to allow environmental microbes to influence the microbial population of interest or permit the murine wound to heal in so far as to not have enough resultant tissue for the study.[17,23] We have also determined that murine wound closure is slightly delayed in the human slough-infected groups compared with non-infected controls (data not shown), an effect that was not statistically significant at day four.[23]

Wound tissue was homogenised in 2 ml tissue grinding tubes with 2.4 mm metal beads and 1 ml PBS and homogenised (Precellys 24) at 5000 rpm for 60 seconds at 25 °C. Wound homogenate was serially diluted and plated on tryptone soy agar with sheep blood medium aerobically for 24–48 hours at 37 °C to determine viable bacterial load in CFU/g. We used one mouse for each human wound sample; one control mouse was included for every four or five infected mice. Human wound samples were used within 24 hours of collection. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (protocol number 07044).

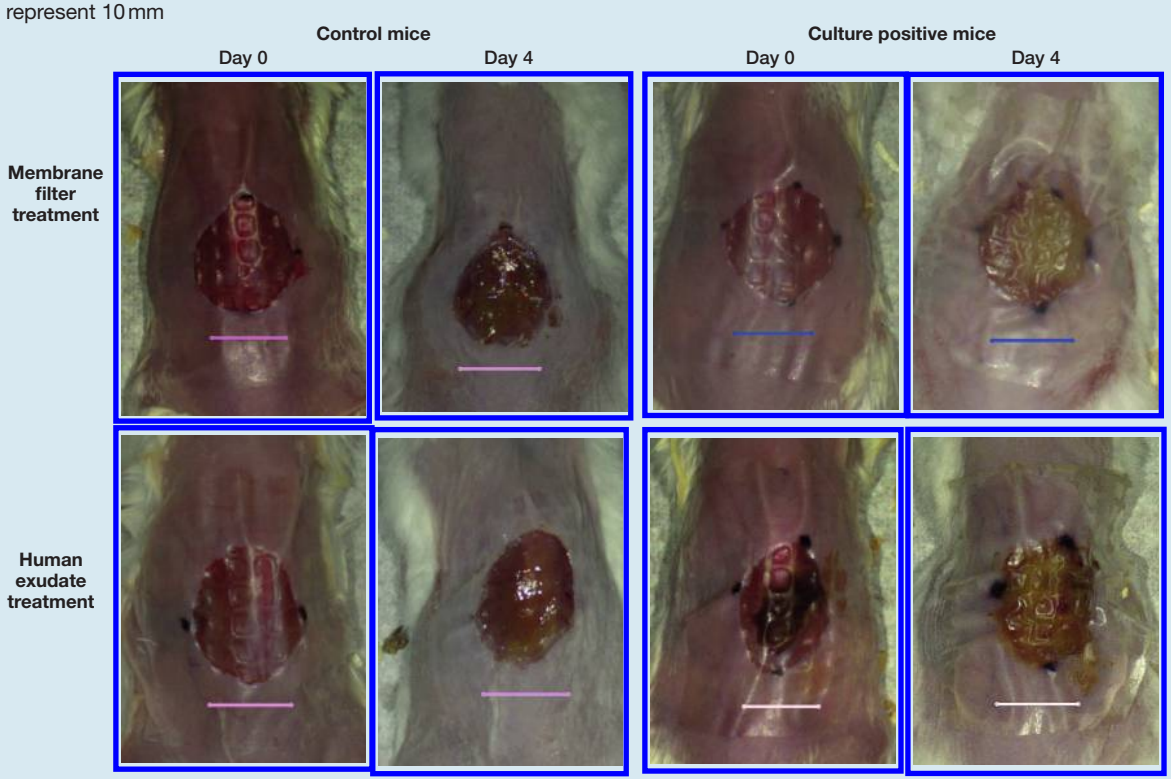
PMA treatment

All samples submitted for PMA analysis were divided in half (PMA-treated and untreated) for performing the live-dead assay. Samples were added to 0.65 ml microtubes (Diagenode Bioruptor, Diagenode Inc., Belgium, Europe), resuspended in 1×PBS, and sonicated on ice using a Diagenode Bioruptor for 12 minutes. Following sonication, 400 µM PMA was added to the PMA treatment samples. Both treated and untreated samples were incubated in the dark at 4 °C for 10 minutes with frequent vortexing. Samples were then exposed to light for 15 minutes using a PMA-Lite LED photolysis device to cross-link the PMA dye to DNA (Biotium, Hayward, CA, US). The percentage of viable cells was calculated by dividing the copy number of PMA-treated bacteria by the total copy number of bacteria.

DNA extraction and quantitative-PCR

Genomic DNA was extracted from samples using the PowerMag Soil DNA Isolation Kit optimised for the KingFisher platform (Mo Bio Laboratories, Inc., Carlsbad, CA, US) according to the manufacturer's protocol. Quantification of total 16S gene copies was performed using the LightCycler 480 (Roche Life Sciences, Indianaoplis, IN, US). Forward

Fig 2. Active and passive transfer of wound microbiota. Both membrane filter treatments and slough collected from patient wounds resulted in highly exuding chronic infections in the mouse model compared with control mice. Scale bars represent 10 mm



(5′-CCATGAAGTCGGAATCGCTAG-3′) and reverse (5′-GCTTGACGGGCGGTGT-3′) 16S primers (20 µM each) were used along with a 16S probe (5′-TACAAGGCCCGGGAACGTATTCACCG-3′) in Quanta qPCR Tough Mix (VWR, Radnor, PA). Template DNA (2.5µl) was loaded into the master mix containing the primers and probe (10µl each) and run with the following thermal cycling profile: 95 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds, 52 °C for 40 seconds, and 72 °C for 1 minute; and final extension at 72°C for 10 minutes. Copy numbers were determined from quantification cycle (Cq) values.

Sequencing

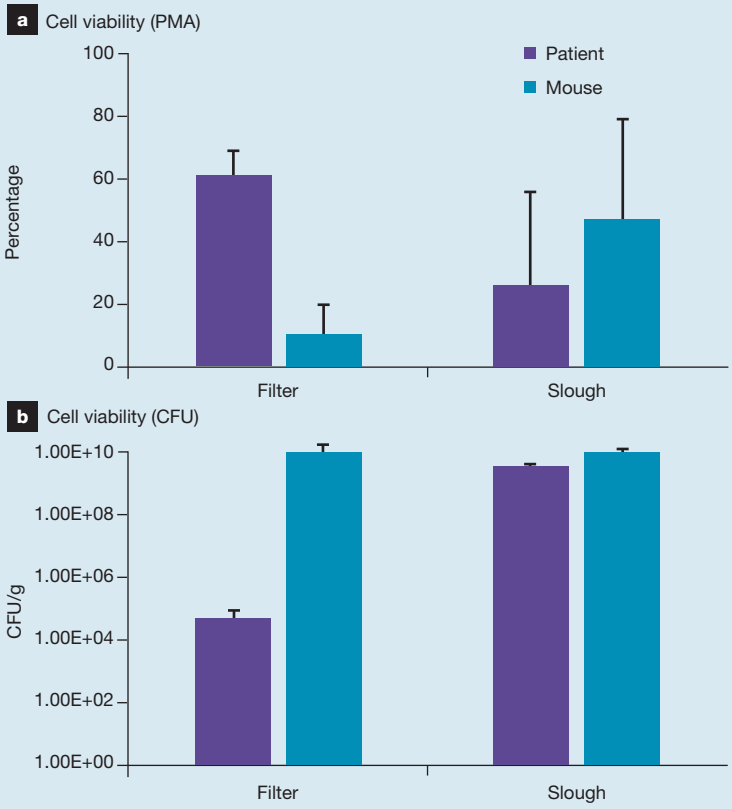
Samples were amplified for semi-conductor sequencing using a forward and reverse fusion primer. The forward primer was constructed with the Ion A linker (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′), an 8–10bp barcode, and the 28F primer (5′-GAGTTTGATCNTGGCTCAG-3′). The reverse fusion primer was constructed with a biotin molecule, the Ion P5 linker (5′-CCTCTCTATGGGCAGTCGGTGAT-3′), and the 388R primer (5′-TGCTGCCTCCCGTAGGAGT-3′). Amplifications were performed in 25µl reactions with Qiagen HotStarTaq master mix (Qiagen Inc., Valencia, CA, USA), 1µl of each primer (5 µM), and 1 µl of template. Samples were amplified on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA, USA) under the following thermal profile: 95°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 54°C for 40 seconds, and 72°C for 1 minute; 1 cycle of 72°C for 10 minutes; and 4°C hold.

Amplification products were visualised with eGels (Life Technologies, Grand Island, NY, US). Products were then pooled into equimolar mixtures. Each pool was size-selected using Agencourt AMPure XP (Beckman Coulter, Indianapolis, IN, US) following Life Technologies protocols. Size-selected pools were quantified using a Qubit 2.0 fluorometer and the Qubit High Sense kit (Life Technologies) and then diluted to 23pM. Diluted pools were subjected to emPCR, enriched using the OneTouch2 system, and sequenced using the Ion Torrent PGM following manufacturer protocols (Life Technologies).

Bioinformatics

The sequence data were then analysed at RTL Genomics (Lubbock, TX) using their standard microbial diversity analysis pipeline. The data analysis pipeline consisted of two major stages, the denoising and chimera detection stage and the microbial diversity analysis stage. Denoising is performed by various techniques to remove short sequences, singleton sequences, and noisy reads. Once the bad reads are removed, chimera detection is performed to aid in the removal of chimeric sequences. Finally, the remaining sequences are corrected base by base to help remove noise from within each sequence. During the diversity analysis stage, each sample is run through the analysis pipeline

Fig 3. Viability of wound microbiota. Using two independent methods to determine cell viability of wound microbiota. While the traditional culture methods, which determine CFU/g, are less variable, they are also vulnerable to false negatives due the presence of unculturable microbial species. Alternatively, the PMA method is much more variable but can account for unculturable species.



to cluster reads into operational taxonomic units (OTUs), which then go through taxonomic classification down to species-level identification. Bacterial species bar plots and tables were created from the 16S sequencing data using R statistical software (R Development Core Team, Auckland, New Zealand).

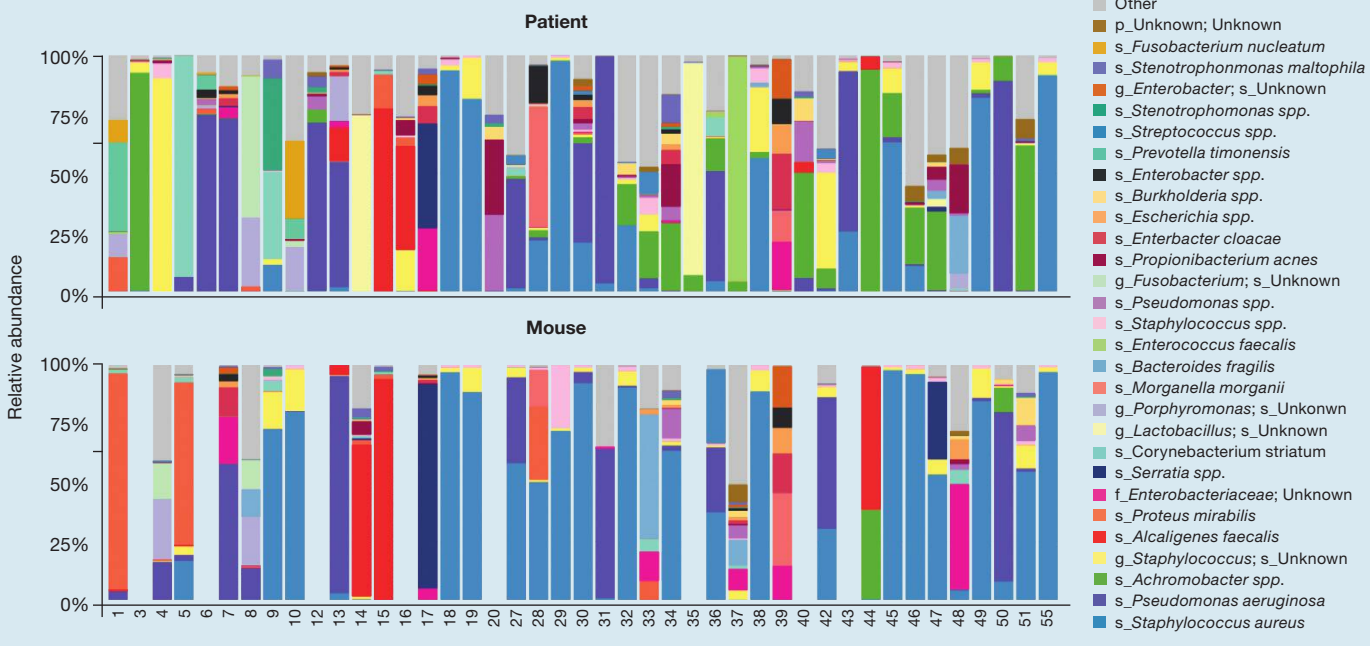
Results

In the chronic wound mouse model, culture-positive mice did show infection, and the wounds became highly exudative by 4 dpi compared with the control mice (Fig 3). The control mice failed to develop measurable microbiota after four days, as evidenced by the absence of observable slough/exudate and negligible CFUs or microbes identified by 16S sequencing. Wound perimeter or area did not differ significantly between control and infected mice at 4 dpi (data not shown). This could be due to the genetic variability of the wound microbiota or mice, or more likely, the short time-frame of the study. The observations were made only till 4 dpi because that was the minimum amount of time needed to produce observable chronic infection in the wound mouse model. To confirm that the resulting infections were due to the transfer of patient wound microbiota into the mouse wound rather than other environmental factors, cell viability assays and 16S amplicon sequencing were carried out on mouse and patient samples.

Cell viability assays conducted on saturated filter paper and debris taken from patients and the subsequently inoculated animal model clearly demonstrated that wound microbiota can leave the wound bed as viable cells and infect new tissues in permissive environments. Cell viability assays using PMA-PCR in all treatment groups demonstrated the presence of viable cells (Fig 3a). Analysed filter paper and slough from patients contained an average of 19% and 25% viable cells, respectively. These percentages are low which may be due to biofilm matrix shielding viable bacterial cells from the PMA stain. Alternatively, filter and slough samples from mouse wounds contained an average of 17% and 47% viable cells, respectively. While slough samples contained more viable cells compared with filter paper samples, both were sufficient to cause infection in a secondary host. These findings were confirmed using traditional culture methods (Fig 3b). Owing to the nature of the study, simply reproducing infection using viable cells as inoculant does not confirm that the initial wound microbiota causes the resulting infections in the mouse model. 16S amplicon sequencing of patient and mouse samples demonstrated that microbial diversity and relative abundance were strikingly similar, with the top 30 species present in both patient and mouse samples (Fig 4).

Since wound slough was shown to contain more viable bacterial cells than filter paper did, i.e., active versus passive dissemination, respectively, slough-induced infections were studied in further detail. 16S amplicon sequencing definitively illustrated that the wound microbiota was not significantly altered through the process of sampling, re-inoculation into a secondary host tissue, and final re-isolation (Fig 5). All 43 non-excluded subjects had samples of wound bed slough submitted for analysis and for seeding onto mouse wounds. The slough of each human chronic wound prior to seeding in the mouse model was analysed, and the wound microbiota (species and relative abundance) was documented. Of the slough seeded onto mouse wounds, 81% (35/43) were positive for polymicrobial communities within the wound from 16S amplicon sequencing (on the basis of culture and 16S data 91% (39/43) showed the propagation of microorganisms within 4 dpi). Not surprisingly, the most prevalent species found within the wound microbiota of patient and mouse samples were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Achromobacter spp.*, and other *Staphylococcus spp*. (Fig 5). Of interest is the finding that 93% of microbes identified in humans were able to propagate in the mouse model. There did not seem to be a specific noteworthy selective pressure for any species (Table 2). These results also demonstrate that the microbiota of the original wound contained significant amounts of viable microbes.

Fig 4. Relative abundance of the top 30 species in patient and mouse wound microbiota



Discussion

The present study was organised with two specific arms to address the different concerns of evaluating active and passive transfer of viable infectious microorganisms. For the first arm, a subset of 25 patients underwent application of Adaptic Touch dressing with filter paper for the collection of exudate and slough. The filter paper was subsequently used for evaluation of viability and infectivity via PMA-PCR, CFU quantification, and mouse models. In the second arm, slough wound microbiota were harvested from the original group of 25 (slough/exudate) and an additional 25 patients (slough only) from the surface of the wound at the interface of the viable host wound bed, which exhibited slight capillary bleeding.

This modern recapitulation of Koch's postulates demonstrates that wound microbiota is fully capable of propagating on new host tissues with no significant alterations in microbial diversity or relative abundance. Since the exudate collected within a filter paper, which never directly contacted the wound bed, demonstrated viable microbes (Fig 3), we established active movement from the wound bed of infectious microorganisms. This is important on several accounts. First, the entire skin surface of each patient with a chronic wound tends to be continuously and actively seeded with wound microbiota, which increases the risk of infection for any elective surgery. Second, any dressing will be seeded with microbes; thus, combination with a nutrient source in the form of exudate risks biofilm formation in the dressing, thereby facilitating the release of harmful products back onto the wound. Finally, there are far-reaching implications in terms of contamination for caretakers, homes, and hospitals.

Infection at its most fundamental level is simply a process of microorganisms inflicting harm on the host. Many different microbial strategies inducing a wide spectrum of host responses have been identified thus far.[24] The most widely applied classification for infection in the clinical setting is acute versus chronic. Acute infections almost invariably display the cardinal signs of Celsus, namely, rubor, dolor, calor, and tumour.

Fig 5. Average relative abundance of wound microbiota in patient and mouse samples

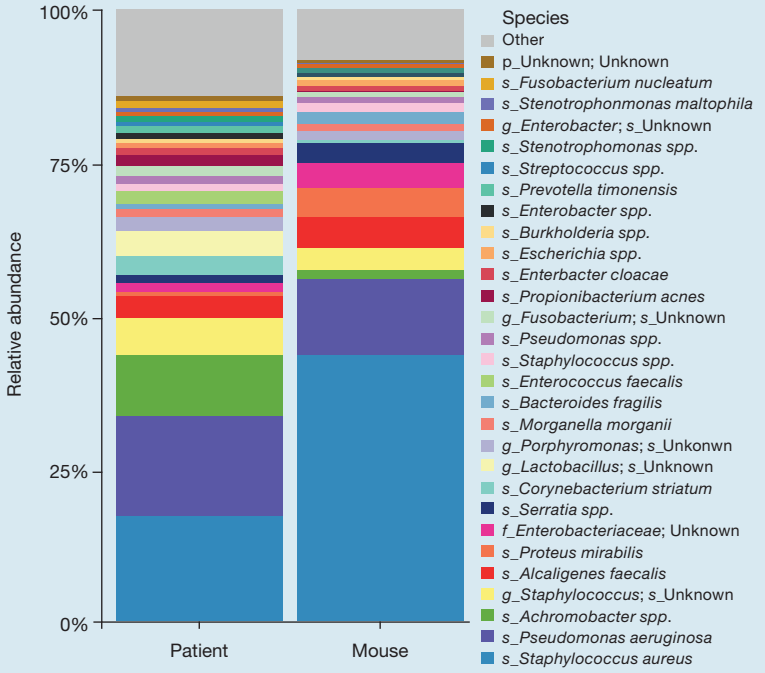


Table 2. Relative abundance of the top 30 species in patient and mouse wound

Patient species relative abundance		Mouse species relative abundance	
(%)	Patient top 30 species present	Mouse top 30 species present	(%)
17.03	s_*Pseudomonas aeruginosa*	s_*Staphylococcus aureus*	42.93
15.58	s_*Staphylococcus aureus*	s_*Pseudomonas aeruginosa*	12.56
10.14	s_*Achromobacter spp.*	s_*Proteus mirabilis*	5.22
5.84	g_*Staphylococcus*; s_Unknown	s_*Alcaligenes faecalis*	4.93
4.18	g_*Lactobacillus*; s_Unknown	f_*Enterobacteriaceae*; Unknown	3.91
3.65	s_*Alcaligenes faecalis*	g_*Staphylococcus*; s_Unknown	3.65
3.61	s_*Corynebacterium striatum*	s_*Serratia spp.*	3.48
2.46	s_*Enterococcus faecalis*	s_*Bacteroides fragilis*	1.94
2.10	g_*Porphyromonas*; s_Unkonwn	s_*Staphylococcus spp.*	1.51
1.87	s_*Propionibacterium acnes*	s_*Achromobacter spp.*	1.38
1.60	s_*Morganella morganii*	g_*Porphyromonas*; s_Unknown	1.20
1.58	g_*Fusobacterium*; s_Unknown	s_*Morganella morganii*	1.19
1.51	s_*Pseudomonas spp.*	s_*Streptococcus pyogenes*	1.00
1.42	f_*Enterobacteriaceae*; Unknown	s_*Escherichia spp.*	0.94
1.30	s_*Prevotella timonensis*	s_*Pseudomonas spp.*	0.90
1.20	s_*Enterbacter cloacae*	s_*Enterbacter cloacae*	0.84
1.16	s_*Serratia spp.*	s_*Streptococcus spp.*	0.83
1.13	s_*Stenotrophomonas spp.*	s_*Burkholderia spp.*	0.75
1.10	s_*Fusobacterium nucleatum*	s_*Corynebacterium striatum*	0.72
1.04	s_*Enterobacter spp.*	g_*Fusobacterium*; s_Unknown	0.70
0.94	s_*Proteus mirabilis*	g_*Enterobacter*; s_Unknown	0.56
0.91	s_*Staphylococcus spp.*	s_*Enterobacter spp.*	0.37
0.86	s_*Stenotrophonmonas maltophila*	s_*Stenotrophomonas maltophila*	0.33
0.76	s_*Bacteroides fragilis*	p_Unknown; Unknown	0.30
0.70	s_*Burkholderia spp.*	s_*Propionibacterium acnes*	0.23
0.68	p_Unknown; Unknown	s_*Stenotrophomonas spp.*	0.16
0.67	g_*Enterobacter*; s_Unknown	s_*Enterococcus faecalis*	0.02
0.62	s_*Escherichia spp.*	s_*Prevotella timonensis*	0.01
0.48	s_*Streptococcus spp.*	g_*Lactobacillus*; s_Unknown	0.00
0.01	s_*Streptococcus pyogenes*	g_*Fusobacterium*; s_Unknown	0.00

However, chronic infection is far subtler. Chronic infection can run the gamut of no significant clinical symptoms to severe exacerbations. It is the broad spectrum of clinical presentations of chronic infections that makes defining a chronic infection so difficult. Currently, most of the criteria used to diagnose chronic infections are clinical signs and symptoms, but the underlying pathophysiology is the presence of biofilm.[13]

Our study included patients with indisputable clinical evidence of chronic wounds of greater than one-month duration, as evidenced by exudate, slough, friable wound bed, tenderness, and most importantly, stalled wound healing (or even progressive worsening). Infection within chronic wounds was demonstrated by using a modern approach to Koch's model for infectious cause and effect. For the purposes of this study, a chronic wound in the mouse model was defined as over 50% of mice demonstrating propagation of wound

microbiota as determined by 16S amplicon sequencing. We observed that 81% of mice inoculated with these human chronic wound samples produced classic chronic wounds. Furthermore, considering mice that showed positive results for 16S amplicon sequencing or that were positive for viable bacteria by CFU enumeration, 91% developed the appearance of chronic wounds. Sampling the mouse chronic wound recapitulated essentially the same polymicrobial milieu that was sampled from the original human chronic wound. It is noteworthy that, although the relative percentage of each species identified in the human wound differed somewhat from that in the mouse wound, the species originally present were reestablished in the mouse model. The Koch model confirms that the wound microbiota is an important, if not primary, causative agent producing a chronic wound.

Evidence-based medicine, which outlines several different criteria for causation, may also be used to ascertain the microbiota's role in the pathogenesis of chronic wounds. The first criterion is association. Microbiota was found to be absolutely associated with chronic wounds 100% of the time, as supported by the 16S amplicon data in the present patients. It must also be remembered that, relative to chronic infections, there are negligible amounts of microbiota in acute wounds that heal as expected.[25] In the present study, we also found consistency and reproducibility of the microbiota across not only human chronic wounds but also a murine wound model. The introduction of microbes into animal wound models produces the distinct characteristics (exudate, delayed healing, slough formation, etc.) in a reproducible manner.[23,26–28] The criteria of 'specificity' are similar in that the overwhelming presence of microbiota is very specific for the chronic wound, and there is no other likely local explanation for the chronicity. Often, host impairments such as endothelial cell dysfunction, microcirculatory impairment, ischaemia, and repetitive trauma are offered in explanation for the chronicity of a given wound. However, if the wound bed is biopsied, the biopsy site will usually quickly heal to the level of the wound bed despite these host impairments. In addition, satellite wounds caused by tape tears or other traumas often heal in two weeks, like acute wounds, despite the same host impairments that the chronic wound is undergoing. Finally, often, a course of antibiotics turns the wound around, and it heals normally even after the antibiotics are withdrawn, although the host impairments remain. Therefore, something specific to the wound bed, such as the microbiota, is the most likely explanation for the chronicity of a wound.

Evidence-based medicine also requires that the cause be temporally associated with the effect. In this study, the control wounds that did not have microbiota seeded onto the wound did not produce excessive exudate, whereas the inimical chronicity in the mouse model was only noted 24–48 hours after seeding with microbiota. Therefore, the close temporal association between seeding and development of an exudative wound demonstrates the microbiota-chronicity cause-and-effect temporality.

Although not addressed in this study, the criterion of a 'biological gradient' is well-understood in medicine in relation to wounds. A highly 'contaminated' wound is considered more at risk of becoming chronic, warranting more aggressive antimicrobial management than that for a 'clean' wound. Therefore, microbiota causing a greater incidence of effect is reflected by a high number of microbes, which fulfils the biological gradient criterion.

No criteria are more important than the plausibility of the mechanism of causation. The European Infectious Disease Society has stated that biofilm is the cause of chronic infections.[29] In the same guidelines, the authors proceed to use chronic wounds as their model of chronic infection. The molecular mechanisms and subcellular pathways of biofilm infection are well-defined.[30–35] Biofilm phenotype bacteria have the ability to attach and propagate on host tissue and secretory systems that can diffuse and/or directly inject molecules into wound bed cells that render them senescent.[36] Biofilms have multiple strategies to produce inflammation, thereby producing plasma exudate from inflamed wound bed capillaries for sustaining nutrition sources.[37] The plausibility that the wound microbiota can produce a chronic infection in a wound is well-established at every level.

There are several other criteria in evidence-based medicine, such as coherence (corroboration of the likelihood of the cause and effect by laboratory findings), experimental data (experimental procedures providing evidence for the cause and effect; Koch's model), and analogy (consideration of similar factors from other 'diseases'). This study replicated Koch's postulates, demonstrating that the microbiota of the human chronic wound is a causative factor for the chronicity of human chronic wounds. Coherence was demonstrated because the laboratory findings of chronic wounds were produced in animal models. However, most importantly, chronic wounds were found to behave like other chronic infections at the molecular and clinical level, and therefore, the criteria of analogy were met.

It is not important in evidence-based medicine that all of Hill's criteria of causation be met. Yet, in this study and the supportive evidence provided, microorganisms could be considered the causative factor for the chronicity of cutaneous wounds on the basis of modern criteria.

Limitations

Limitations to our methods included protocol variations resulting from transport longer than two hours and tissue size insufficient in volume or mass, which led to the exclusion of 12 patients from the filter paper arm and seven from the slough arm of 50 initial patients. Nevertheless, the goal was not statistical

significance, but rather to prove the possibility of viable microbial transfer through exudate. Therefore, we expect that these limitations did not severely impact our findings.

Conclusion

The null hypothesis for this study was that the wound microbiota does not cause a chronic wound when transferred to another healthy host. Indeed, eight mice failed to develop a chronic wound when seeded with a sample of human slough. Regardless, since the overwhelming majority of mice (81%) did develop a chronic wound and none of the controls developed a chronic wound, the null hypothesis is rejected. The most likely explanation for the eight failures of mouse wounds to become chronic lies in our collection, transfer, culture, or seeding methods rather than the inability of the wound microbiota itself to infect.

This study answers many controversial questions about the role of microorganisms in the chronicity of human cutaneous wounds (Table 1). The microbiota of chronic wounds is mostly viable and retains the ability to reestablish itself (infect) in a permissive host environment. In a mouse model, a wound that is not exposed to microorganisms heals normally, whereas wounds seeded with a polymicrobial mixture of microorganisms directly from a human chronic wound acquires characteristics of a chronic wound. Both the Koch model and evidence-based medicine criteria for causation were fulfilled, implicating the wound microbiota as an important cause of the chronicity of wounds.

Chronic wounds present a spectrum of clinical presentations, which appears to be related not only to the microbial species or to host factors but also to the synergies of microbial strategies used for infection. Microbial phenotype very likely plays an important role. As a cause for infection within clinical chronic wounds, wound biofilm might be an important consideration in the pathogenesis of chronic wounds. Further investigations will be necessary to elucidate the molecular mechanisms by which biofilm as a polymicrobial unit might produce chronic wounds. However, until more is known, directly addressing the wound microbiota in every chronic wound needs to be an anchoring principle for the management of chronic wounds. **JWC**

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