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ORIGINAL ARTICLE

Bacteria, biofilm and honey: A study of the effects of honey on 'planktonic' and biofilm-embedded chronic wound bacteria

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Abstract
Chronically infected wounds are a costly source of suffering. An important factor in the failure of a sore to heal is the presence of multiple species of bacteria, living cooperatively in highly organized biofilms. The biofilm protects the bacteria from antibiotic therapy and the patient's immune response. Honey has been used as a wound treatment for millennia. The components responsible for its antibacterial properties are now being elucidated. The study aimed to determine the effects of different concentrations of 'Medihoney™' therapeutic honey and Norwegian Forest Honey 1) on the real-time growth of typical chronic wound bacteria; 2) on biofilm formation; and 3) on the same bacteria already embedded in biofilm. Reference strains of MRSE, MRSA, ESBL Klebsiella pneumoniae and Pseudomonas aeruginosa were incubated with dilution series of the honeys in microtitre plates for 20 h. Growth of the bacteria was assessed by measuring optical density every 10 min. Growth curves, biofilm formation and minimum bactericidal concentrations are presented. Both honeys were bactericidal against all the strains of bacteria. Biofilm was penetrated by biocidal substances in honey. Reintroduction of honey as a conventional wound treatment may help improve individual wound care, prevent invasive infections, eliminate colonization, interrupt outbreaks and thereby preserve current antibiotic stocks.

Introduction

Chronic wound care is costly and difficult. It is estimated that 1% of the UK and US populations have a chronic sore, with wound care costs in Britain alone estimated at £1 billion per y [1]. The chronic sore is often a painful, exuding, malodorous portal for invasive infection and a reservoir for antibiotic resistant bacteria, capable of causing outbreaks.

Honey has been used as a wound treatment for millennia [2]. It is once again being recognized that honeys of differing botanical provenance have varying levels of antibacterial and wound healing activity [2]. In 1991 a survey of the antibacterial activity of local honeys was carried out in New Zealand [3]. This has lead to the licensing of standardized, sterilized Leptospermum plant species honey as a wound treatment. The granting of a licence was a pivotal moment, as it allows for reproducible clinical trials and in vitro experiments. Several studies have now confirmed the bactericidal nature of therapeutic honeys against many species of bacteria, including strains resistant to antibiotics [4,5]. However, most in vitro honey studies have used methods traditionally used for assessing the likely antibiotic sensitivity of bacteria to serum levels of antibiotics – the reporting of minimum inhibitory concentration on agar plates. Honey, meant for topical application, is not itself an antibiotic, but a complex mixture of substances with biocidal effects [2]. Therefore, biokinetic studies of bacterial growth related to concentration of biocide through time, may be more appropriate [6]. A further difficulty of generalization of results from in vitro studies conducted on agar plates, is that bacteria are tested in their most vulnerable 'planktonic' form. Bacteria associated with chronic infections most often live in a biofilm – a highly organized, slimy, bacteria-secreted polysaccharide layer attached to a surface, be it the patient's tissue or a prosthetic part [7,8]. The biofilm protects the bacteria from the

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patient's immune system, antibiotics, drying and washing. The more vulnerable planktonic form occurs only fleetingly, to enable spreading of the colony [7]. Within the biofilm, the characteristics of the bacteria change, so that biofilm-embedded bacteria are up to 1000 times more resistant to antibiotics than the 'planktonic' bacteria that are used in microbiology laboratories to test antibiotic sensitivity [7].

Understanding the biofilm survival strategy is crucial to understanding the failure of adequately perfused, non-malignant sores to heal. Where the wound is 'critically colonized' (without signs of systemic infection), topical agents must combat the biofilm if healing is to progress [8]. It has long been recognized that the greater the number of bacterial species isolated from a wound, the more recalcitrant the wound [9]. Synergy between different bacterial species living cooperatively in biofilm explains this increased resistance to healing [9].

The aim of this study was to use new techniques to test the effects of honey on typical, difficult-to-treat wound bacteria, in their planktonic and biofilm-embedded states. We also hoped to find a Norwegian source of honey that might, with proper standardization and sterilization, prove a relatively cheap, adequately antibacterial solution to the costly problems of antibiotic-resistant wound bacteria.

The study had 3 phases. First, we used a real-time broth microtitre plate technique to compare the effects of Medihoney™ with a Norwegian Forest Honey on the growth of typical wound bacteria. The ability of each bacterial strain to form biofilm when exposed to the honey dilution series was then assessed. Next, controlled inocula of the bacteria were allowed to grow biofilm in a microtitre plate, before being incubated with the honey dilution series.

Materials and methods

Honeys

'Medihoney™' (Medihoney Pty Ltd., Queensland, Australia) is a commercially available medically licensed preparation for wound dressing (European Union certification as a medical device (class IIb) for wound care). Medihoney consists of a standard mixture of gamma-irradiated antibacterial honeys including Leptospermum species honey. The honey was kept in plastic tubes, excluding light. Gamma-irradiation of honey has been found to kill any spores of Clostridium botulinum, rendering it sterile without damaging the antibacterial enzymatic activity of the honey [10].

The second honey is a commercially available, unmixed forest honey from a small producer (Solhøy Bigård, Østfold, Norway) intended as a food product. The honey was produced during the summer season 2007. The honey is pressed from the honeycombs and filtered to remove wax particles. No heat treatment takes place. The honey was supplied in glass jars. Both honeys were kept at room temperature.

As volumes of 100% honey are difficult to assess, the densities of the two honeys were determined, and 50% (v/v) stock solutions made by weighing and dissolving the honey in the appropriate volume of broth (Nutrient Broth CM0001 (Oxoid, UK), with the addition of 1% glucose). Less than 30 min before inoculation, 2-fold dilutions in broth were made from this stock. After 1:1 dilution with bacterial suspension, the final concentrations of honey used were 25, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4%.

Bacterial strains

Two Gram-positive and 2 Gram-negative bacterial species were selected. The MRSA strain was originally isolated in our laboratory from a pus sample from the paediatric surgery department of Oslo University Hospital, Ullevål. Genetic typing of the MRSA strain was performed at the Epigen Institute (Akershus University Hospital, Norway). Its genotypic markers (SCCmec type IV, MLST 80, Panton-Valentine leucocidin-positive, spa type t044) were identical to that published in a recent report of a serious 'community acquired' infection of a previously healthy young man in Norway [11]. In addition, 3 reference bacterial strains were obtained from the Culture Collection, University of Gothenburg, Sweden: methicillin- resistant Staphylococcus epidermidis MRSE (CCUG 21989), ESBL Klebsiella pneumoniae (CCUG 45421) and Pseudomonas aeruginosa (CCUG 17619).

Bacterial growth assays

The microtitre broth dilution protocol described by Wiegand et al. was employed [12]. Briefly, McFarland standard 0.5 turbidity suspensions (bioMérieux cat. No. 70900), of 1×10^8 colony forming units/ml in sterile saline were prepared from 24-h agar incubations of pure cultures of each bacterial strain. The McFarland 0.5 solutions were then diluted 1:100 in sterile nutrient broth to produce working inocula of 1×10^6 cfu/ml, which, when diluted with the honey preparations reached the desired standard inocula of 5×10^5 cfu/ml. Prepared samples were used within 30 min of preparation. To produce the bacterial 'mix', equal volumes of each of the 4 bacterial species at MacFarland 0.5 turbidity were mixed together and

diluted to 1×10^6 cfu/ml as above. 50-μl volumes each of bacterial inoculum and 50-μl honey dilution were mixed and sown out (in duplicate) in a sterile, polypropylene, round-bottomed, 96-well microplate (Corning Costar, cat. No. 3790). Sterility control wells with broth only and each concentration of honey (100 μl) were included in all experiments. Each bacterial strain was grown in broth without honey to provide a full growth control. The microplates were sealed with optic film (ABsolute QPCR Seal, cat. no. AB-1170, Thermo Scientific UK) to prevent evaporation, and incubated in the Victor 3 Spectrophotometer (Perkin Elmer, Massachusetts, USA) at 35°C, with shaking, for about 20 h. Photometric readings were automatically made every 10 min at 620 nm.

Immediately after sowing out the microplates, samples of each bacterial strain were sown out on blood agar plates as control for the inoculum size and purity, according to the instructions of Wiegand et al. [12].

The growth assay experiments were repeated 4 times on Medihoney and twice on the Norwegian honey. Average growth curves were produced using Excel statistics package (2007, Microsoft Corporation, USA), by plotting the average optical density value at each time interval using data from all experimental rounds.

Minimum bactericidal concentrations of planktonic bacteria

After incubation, all controls without bacteria, and other wells that exhibited no, or little growth on the growth charts were examined. Aliquots of 50 μl from each sample well were diluted with 9 volumes sterile saline and vortexed to wash the honey off the bacteria cells, a method described by Carson et al. [13]. Then, 50 μl of the washed samples were sown out, in duplicate, to blood agar plates and incubated aerobically at 35°C for 20–24 h. The minimal bactericidal concentration (MBC) was taken to be the lowest concentration of honey that killed 99.9% of the original number of bacteria [13].

Examination of biofilm formation

Standard bacterial inocula were prepared as above. Using 1 microplate per bacterial species, each bacterium was sown out with 8 wells per honey dilution (50 μl bacterial inoculum plus 50 μl honey dilution), including 0% honey as a growth control. Four wells without bacteria acted as sterility controls for each honey dilution. The microplates were sealed with optic film, and incubated aerobically at 35°C, with shaking. Optical densities at 620 nm were measured after 24 h to control for bacterial growth.

Visualization of biofilm formed was performed using a method based on that described by Stepanović et al. [14]. After incubation, the microplates were emptied by flicking onto absorbent paper. The microplates were washed 3 times by immersion and agitation in cold tap water, before drying at 60°C for 1 h. The adherent biofilm was then stained by careful pipetting of 50 μl 0.5% crystal violet solution into the bottom of each well. After 20 min at room temperature, the microplates were emptied and washed in running, cold tap water until free of excess stain and then air-dried overnight. Next, the stained biofilm was solubilized by addition of 50 μl 33% acetic acid. After 30 min the optical densities at 600 nm were measured and photographs were taken. Biofilm formation was assessed 4 times for Medihoney, twice for Norwegian honey.

Examination of biofilm sterilization

To meet the aims of the third part of the study, biofilm was grown by sowing out standard inocula (5×10^5 cfu/ml) of the bacterial strains as 50-μl inocula volumes in 50 μl sterile nutrient broth. Two microplates were sown out in this manner per bacterial species, for each honey – 1 destined for biofilm visualization and the other 'sacrificed' for MBC examination of the biofilm-embedded bacteria. A control microplate containing each of the bacterial species was also included, to check the formation of biofilm after 24 h, before addition of the honey. The microplates were sealed and incubated at 35°C with shaking. After 24 h, the optical densities (at 620 nm) were measured of all microplates to check bacterial growth. The microplates were then emptied by flicking onto absorbent paper, and washed 3 times in sterile water by immersion, agitation and flicking onto absorbent paper. Each bacterial species was washed in a separate sterile container to avoid cross-contamination. Microplates then received the honey dilutions, and were incubated as before for a further 24 h. The '24-h biofilm control microplate' underwent biofilm staining.

MBC determinations were performed as described above, but the pipette tip was used to scrape the bottom of the test wells to disrupt biofilm, and a direct sample of unwashed, biofilm-embedded bacteria was included in the blood agar plate checks of 50-μl saline-washed samples.

Biofilm visualization was then carried out as described above. Experiments were repeated 3 times for each honey.

Reproducibility was judged by comparing the MBCs between rounds, overall pattern of growth

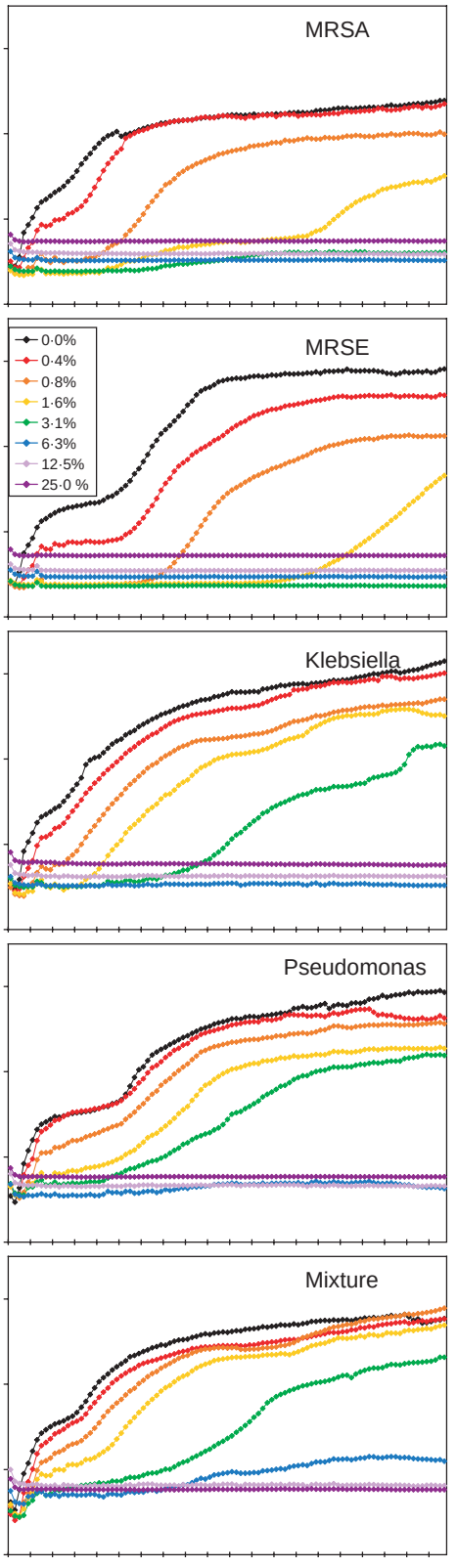

Figure 1. Optical densities at 620 nm during growth of different bacteria in presence of different concentrations of Medihoney. The colours of the curves correspond to the concentrations described in the key. Each x-axis tick represents 1 h. The y-axis scales are 0.1 to 0.8 A620 units with ticks at 0.3, 0.5 and 0.7.

on the growth curves, and the appearance of stained biofilm in the microplates.

Results

Figure 1 presents the effects of Medihoney on the growth of controlled inocula of planktonic bacteria. The average growth curves from 4 experimental rounds are presented. The agar plate MBCs of each honey against bacteria in its planktonic and biofilm-embedded form are presented in Table I.

Both of the honeys were found to slow the growth of exponential phase bacteria from as low as 0.8% honey. The Norwegian forest honey (not shown) produced similar extended lags in bacterial growth with increasing honey concentration. For both honeys, the wells that corresponded to the bactericidal concentrations on agar (Table I) showed no increase in optical density over 20 h on the graphs. It was observed that not a single colony grew on agar at these MBC concentrations. On 1 round, the Medihoney completely killed planktonic Pseudomonas at 6%, and this was reflected on the graph for that round as well as agar plate MBC determination. In 1 of 3 experiments on biofilm-embedded bacteria, the MBC of the Norwegian honey on MRSA was 25%. Otherwise, the agar plate MBCs did not vary between experiments.

When the MBCs were assessed from the partially inhibited bacterial mix, it was observed that the ESBL Klebsiella had dominated the other bacterial species, both in the planktonic and biofilm-embedded forms.

Figure 2 presents the effect of Medihoney and Norwegian forest honey on MRSA biofilm formation. The other bacterial species (not shown) exhibited the same phenomenon – where there was no bacterial growth, there was no biofilm formation. At 3% Norwegian honey, a denser biofilm was formed than at lower concentrations. At 1.6% Medihoney, it appeared that there was still some growth-inhibiting effect (as confirmed by the growth curves), which was overcome at 0.8%, where darker biofilm was observed.

Discussion

The primary aim of this study was to examine whether honey is a useful topical biocide against a range of bacterial species typically found to infect wounds. The results confirm that there are differences in the susceptibility of different bacterial species to honey. However, differences in susceptibilities may exist between different strains of the

Table I. Minimum bactericidal concentrations for planktonic and biofilm-embedded bacteria.

	Planktonic bacteria		Biofilm-embedded bacteria	
	Medihoney™	Norwegian forest honey	Medihoney™	Norwegian forest honey
MRSA	3%	6%	6%	12%[b]
MRSE	3%	6%	6%	12%
ESBL Klebsiella	6%	12%	12%	25%
Pseudomonas	12%[a]	12%	12%	25%
Mix	12%	12%	12%	25%

[a]In 1 of the 4 experiments the MBC of Medihoney on planktonic Pseudomonas was 6%.
[b]In 1 of 3 experiments the MBC of the Norwegian honey on biofilm-embedded MRSA was 25%.

same species, so the results for each strain should not be interpreted as representative of the entire bacterial species.

It is clear from the graphs that the honey inhibited the growth of planktonic bacteria even at very low concentrations. These results confirm previously published agar-plate based studies, but with the advantage of allowing a clear view of the patterns of bacterial growth on a time-scale that may reflect the practical necessities of changing dressings on an exuding wound. Biofilm appeared to offer a degree of protection to MRSA, MRSE and the ESBL Klebsiella, but Table I indicates that the active substances in the honey were able to diffuse through the established biofilm matrix. The Pseudomonas strain produced copious biofilm under the test conditions, but, interestingly enough, remained as vulnerable to Medihoney within the biofilm matrix as it was in a planktonic form.

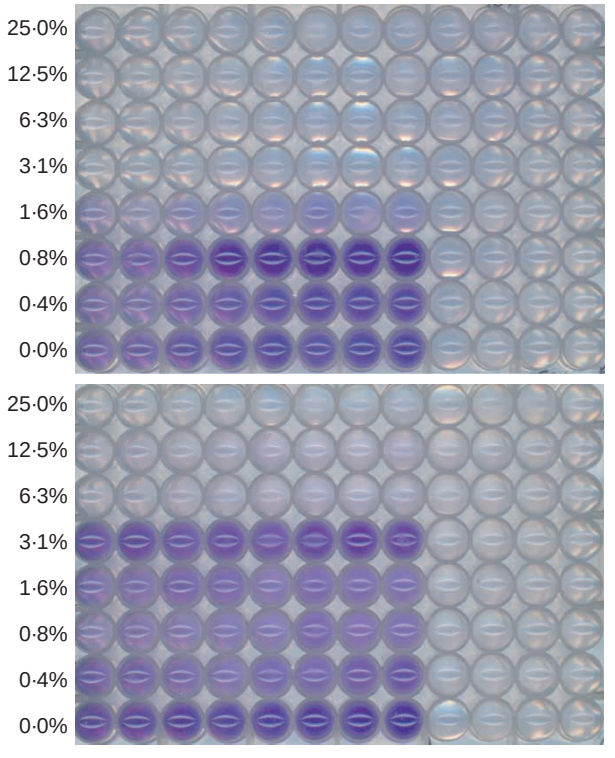

Figure 2. Biofilm formation by MRSA in presence of different concentrations of honey. Top panel: Medihoney. Bottom panel: Norwegian forest honey. Honey concentrations are shown to the left of the panels. The 4 columns to the right in both panels did not contain bacteria. Purple colour represents biofilm-embedded bacteria.

Figure 2 appears to indicate that the honey dilution series presented a balance between substances that were toxic to the bacteria, and sugars that were used as energy source and building blocks for biofilm formation [14].

In the interests of biological clarity, the growth curves are presented without subtraction of the respective control wells that did not contain bacteria. At lower honey concentrations, in the early hours of the experimental run, subtraction of the control values from the bacteria-inoculated well readings resulted in negative absorbance values. This we believe was due to the formation of condensation on the sealing film in the control wells with low honey concentrations. The amount and quality of the condensation differed between those wells containing bacteria and the controls. Higher concentrations of honey did not produce condensation, due to its hygroscopic nature. As no bacteria were ever found to grow from control wells for the honey or broth when sown out on agar, it was decided that subtraction of the control values was not meaningful, especially as it did not change the overall pattern of growth, as presented in Figure 1. The controls thus only functioned as a check on purity/contamination. The slight dips of some of the growth curves at the beginning of the experiment were due to the settling of the well contents by shaking of the instrument.

Over 60 bacterial species have been reported to be susceptible to honey [2]. The overall antibacterial activity of a manuka (Leptospermum scoparium) honey appears to be a complex synergistic effect of methylglyoxal [15,16], hydrogen peroxide (produced by the glucose oxidase added to honey by the bees [17]), sugar components, phenolic compounds including flavonoids (directly reflecting the floral source of the nectar), acids and minerals [2].

An advantage of the multiple modes of action of honey is that the risk for development of bacterial resistance may be decreased [2]. The contributions of each of these active components of the honey may differ at different dilutions, pH and temperature. In the wound the interaction with host factors, dead bacteria and quenching systems in the honey itself must be considered [17].

The sugar content of honey may be important, beyond the osmotic pressure it exerts. Lerrer et al. [18] have shown that fructose in honey prevents establishment of Pseudomonas aeruginosa infection by blocking the bacterial sugar binding protein, or 'lectin', whose natural target is the fucose molecules that are expressed on target tissue cells. The matrix of biofilm is itself a polysaccharide, and it is becoming apparent that sugar molecules are also used as chemical messages between bacterial species [19]. The recent finding of methylglyoxal in significant amounts in Leptospermum species honey is intriguing [15,16]. Methylglyoxal is a highly reactive substance found in the glycolysis pathway of plants, fungi, bacteria and eukaryotes. It is known to act as an inhibitor of cell division in eukaryotic cancer cells [20]. Further investigations of the effects of honey on the cell cycle, and of specific sugars in bacterial adhesion, biofilm structure and bacterial communication may be useful in producing new classes of antibacterials.

The composition of the Norwegian forest honey is not known. While not being quite as effective as Medihoney, it was also bactericidal. The botanical provenance is likely to be typical Norwegian forest flowers and honeydew (the sap of pine trees that is ingested by aphids and excreted upon the tree, before collection by the bees). As food honeys are not sterile it is not recommended that they be used for wound care. It has been noted that the most active Leptospermum species honeys, containing the highest concentration of methylglyoxal, come from the harshest climatic areas in New Zealand [15,16]. Elucidation of the origin of the methylglyoxal, and further investigations of honeys from Norway and around the world may prove fruitful.

The results of clinical trials with standardized, sterilized honeys are now being published. Johnson et al. found Medihoney™ to be as effective as mupiricin in preventing bacteraemia from intravenous portals in an intensive care unit [21]. A 3-y trial of honey in wound care at the Department of Paediatric Haematology and Oncology, Children's Hospital, University of Bonn reports: "In our experience, it is possible to guide the oncological patient with a chronic wound through a high-dose chemotherapy with autologous stem cell transplantation without secondary complications if Medihoney is used for wound care." [22].

MRSA incidence rates are increasing in Norway, as in the rest of the world. Where prevalence of antibiotic resistance is increasing, there is a tendency for the clinician to 'seek security in polypharmacy' [23], thus worsening the situation further. 'Community acquired' MRSA infections are typically soft tissue and skin infections, amenable to topical treatment [5]. Reintroducing clinical grade honey for wound care may be an effective part of a strategy to eliminate skin colonization, improve individual wound care, prevent invasive infections from wounds, interrupt costly outbreaks and thereby preserve current antibiotic stocks.

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