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ORIGINAL RESEARCH ARTICLE

Testing the susceptibility to manuka honey of streptococci isolated from wound swabs

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Summary

Honey is a broad spectrum antimicrobial agent that has been re-introduced into clinical practice to treat wounds. Wounds support polymicrobial communities of bacteria that either colonise or infect wounds. Strains with resistance to antibiotics are difficult to eradicate and pose a risk of transfer to other patients. Manuka honey has been shown to inhibit many of the bacteria commonly associated with wounds, such as staphylococci, pseudomonads, coliforms and anaerobes, but its efficacy against streptococci isolated from wounds has not been reported. Using macro- and micro-dilution in broth and an agar incorporation technique, the susceptibility to manuka honey of 15 cultures of catalase negative, Gram positive cocci that had been isolated from wounds was tested. All cultures were inhibited by 10% (v/v) manuka honey and statistically significant differences between the three test methods were not found. Manuka honey offers clinical potential in eradicating streptococci from wounds.

Keywords: streptococci, wounds, manuka honey, susceptibility testing.

Introduction

Honey was used extensively as a medicine by ancient civilisations. Evidence from artefacts such as engraved tablets and drawings, as well as religious, secular and medical texts indicates how it was incorporated into treatments that were applied to the surfaces of wounds more than 4500 years ago (Crane, 1999). Although wound infections are largely treated with antibiotics in conventional medicine, modern wound care products containing honey are now available in many countries and the therapeutic properties of honey are being appreciated once again. The range of curative properties attributed to honey that are pertinent to wounds includes antimicrobial activity and the ability to promote effective healing (Molan, 1999).

An extensive review of the antimicrobial activity of honey showed it to be derived from high sugar content, low water content, acidity, the generation of hydrogen peroxide on dilution and phytochemical components (Molan, 1992). More recently methylglyoxal was discovered to contribute to the activity of New Zealand's manuka honey (Mavric *et al*, 2008; Adams *et al*, 2008), bee defensin-1 was detected in a Dutch honey (Kwakman *et al*, 2010) and melanoidins were identified in Canadian honeys (Brudzynksi *et al* 2010). Hence there is diversity in the components that contribute to the antimicrobial characteristics of natural honeys and the importance of floral origin is not yet fully understood. Although many studies into the antimicrobial activity of honey have been published, the various methods utilised and the different kinds of honey tested has made comparison of reported data difficult (Molan, 1992).

Wounds are colonised by a wide variety of micro-organisms and the bacteria most commonly associated with wound infections are staphylococci, streptococci, coliforms, pseudomonads and anaerobes (Bowler, Duerden and Armstrong, 2001). Reports of laboratory studies demonstrating the inhibition of potential wound pathogens by honey testify to its broad spectrum of activity (Willix, Molan and Harfoot, 1992; Cooper, Molan and Harding, 2002; Cooper, Halas and Molan 2002; Al-Waili 2004; French, Cooper and Molan, 2005; George and Cutting, 2007; Kwakman *et al*, 2008; Blair *et al*, 2009; Tan *et al* 2009; Matjan *et al*, 2010; Sherlock *et al*, 2010).

Although susceptibility of oral streptococci to honey has been investigated (Basson, du Toit and Grober, 1994; Steinberg, Kaine and Gedalia, 1996; Basson and Grobler, 2008; Badet and Quero, 2011), no studies have focused on the susceptibility of streptococci obtained from wounds to honey, there being just two reports of a single strain being tested (Willix, Molan and Harfoot, 1992; Al-Wailii, 2004). This study was designed to address this deficiency.

Materials and methods

Cultures used

The test bacteria utilised in this study were either isolated from wound swabs collected from out-patients attending clinics managed by the Wound Healing Research Unit at Cardiff Royal Infirmary or were provided by the late Alan Paull from wound swabs being processed in the Department of Medical Microbiology and Public Health Service Laboratory at the University of Wales Hospital in Cardiff. For this study the organisms were labelled 1 to 15.

Isolates were subcultured onto blood agar plates (Oxoid, Basingstoke, UK) at two weekly intervals during the study. The identity of the organisms was determined by Gram stain, catalase test, Lancefield group (Streptex test, Oxoid) and biochemical reactions (API 20 Strep, bioMérieux, Basingstoke, UK). Streptex and API 20 Strep kits were used according to the manufacturer's instructions.

Antibiotic sensitivity

A modified Kirby-Bauer technique was used to determine antibiotic susceptibility. Lysed horse blood agar plates were utilised, with antibiotic sensitivity disks obtained from Oxoid and incubation conditions of 37°C for 24 hours. Concentrations of the antibiotics were as follows: penicillin (P) 10 iu, tetracycline (T) 10 µg, erythromycin (E) 5 µg, trimethoprim (W) 5 µg, gentamicin (CN) 10 µg and sulphafurazole (SF) 300 µg.

Honeys utilised

A sample of manuka honey with non-peroxide antibacterial activity equivalent to 18% (w/v) phenol was used (Allen, Molan and Reid, 1991). For comparison an artificial honey solution was prepared as described previously (Cooper, Molan and Harding, 2002).

Minimum Inhibitory Concentration determined by broth macro-dilution in Kahn tubes

Assuming the density of honey to be 1.37 g/mL (for weighing out the honey), a stock solution of 20% (v/v) manuka honey was prepared in sterile deionised water and used to prepare a range of honey concentrations varying by 1 %(v/v) intervals in Kahn tubes by diluting with sterile double strength Todd Hewitt broth (THB; Oxoid, Basingstoke, UK). Total volume in each tube was 2 mL. Tubes were inoculated with 4 µL overnight culture of test organism grown in THB (typically 5 x 10^6 cfu) and tubes were incubated at 37°C for 24 hours and observed for turbidity. The lowest concentration of honey in THB that prevented turbidity was recorded as the Minimum Inhibitory Concentration (MIC).

Minimum Inhibitory Concentration determined by broth micro-dilution in microtitre plates

As described above a range of concentrations of manuka honey in THB was prepared, except that the total volume in wells of 96 well microtitre plates (Nunc, Roskilde, Denmark) was 200 µL. Wells were inoculated with 1 µL an overnight culture of test organism as above and incubated at 37°C for 24 hours. Turbidity was assessed using a plate reader (Anthos Labtech Instruments, Wals, Austria) at 492 nm. For each microtitre plate, one row of wells contained serially diluted honey in THB without inoculum; absorbance at 492 nm due to each dilution of honey in broth was measured and subtracted from that of respective inoculated wells measured after brief shaking to resuspend bacteria. Hence any increase in absorbance due to bacterial growth was detected and the lowest concentration (MIC) preventing growth of each isolate was determined. The test was done on three separate occasions.

Minimum Inhibitory Concentration determined by agar incorporation method

Stock solutions of 20% (v/v) manuka honey and 60% (v/v) artificial honey were prepared in sterile deionised water and used to prepare a range of honey concentrations varying by 1% (v/v) intervals by mixing an equal volume of honey solution at twice the final concentration with molten double strength tryptone soya agar (Oxoid, Basingstoke, UK). The total volume of agar and honey in each plate was 20 mL. Plates were made immediately before use, allowed to set and were dried for 15 min at 37°C before being inoculated with 1 µL overnight broth culture of each test organism grown in THB using a multipoint inoculator (Mast Diagnostics Ltd, Merseyside, UK). They were incubated at 37°C for 24 hours and observed for growth. The lowest concentration of honey that prevented growth was recorded as the MIC. Tests were performed one to three times and plates were prepared in duplicate.

Results

Characterisation of wound isolates

All test organisms were found to be catalase negative Gram positive cocci arranged in chains. Three isolates were found to be *Enterococcus faecalis* and 12 were pyogenic, beta haemolytic streptococci, mostly of Lancefield group G (Table 1). Resistance to the antibiotics tested was detected more frequently in the entercocci than in the streptococci.

Table 1. Characterisation of wound isolates

Culture number	Lancefield group	Name	Antibiotic sensitivity					
			P	T	E	W	CN	SF
1	A	*Streptococcus pyogenes*	S	S	S	S	R	R
2	C	*Streptococcus equisimilis*	S	S	S	S	R	S
3	D	*Enterococcus faecalis*	R	R	R	S	R	R
4	D	*Enterococcus faecalis*	R	R	R	R	R	R
5	D	*Enterococcus faecalis*	R	R	R	R	R	R
6	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	S	S	R	R
7	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	S	S	R	S
8	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	S	S	R	R
9	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	R	S	R	R
10	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	S	I	R	R
11	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	S	R	R	R
12	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	R	I	R	R
13	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	S	S	R	S
14	G	*Streptococcus dysgalactiae* subspecies *equisimilis*	S	R	S	S	R	R
15	G	*Streptococcus porcinus*	S	R	S	S	R	R

P = penicillin 10 iu, T= tetracycline 10µg, E= erythromycin 5 µg, W= trimethoprim 5 µg, CN= gentamicin 10 µg and SF= sulphafurazole 300 µg. S= sensitive; I=intermediate resistance; R= resistant.

Susceptibility of clinical isolates to manuka honey

Susceptibility of the cultures isolated from wounds to manuka honey was determined by three methods (Table 2) and MICs ranged from 5 to 10% (v/v).

Mean MICs for all isolates were found to be 8.2, 7.5, 7.3% (v/v) manuka honey using Kahn tubes, microtitre plates and agar plates, respectively. Using the Kruskal-Wallis test (Minitab version 15) no statistically significant differences were found. MICs for artificial honey determined with the agar incorporation method showed the susceptibility of the isolates to be markedly lower than those obtained with manuka honey indicating that the inhibitory activity of manuka honey was not confined to the main constituent sugars.

Discussion

The taxonomy of the Gram positive, catalase negative cocci has undergone several revisions, with separation of *Streptococcus* and *Enterococcus* in 1984 (Facklam, 2002). Although some species of

Table 2. Susceptibility of clinical isolates to manuka honey.

Culture number	Mean Minimum Inhibitory Concentration of honey (% v/v)			
	Manuka honey in Kahn tubes (n=1)	Manuka honey in microtitre plates (n=3)	Manuka honey in agar (n*)	Artificial honey in agar (n=1)
1	9	7.7	8.8 (6)	23
2	8	8.3	7.3 (4)	19
3	8	7.0	7.7 (6)	15
4	10	8.3	8.0 (2)	>30
5	10	8.7	9.3 (6)	>30
6	7	7.0	6.8 (6)	20
7	9	8.0	9.0 (4)	>30
8	10	8.3	8.0 (4)	24
9	7	7.0	7.3 (4)	21
10	8	6.7	5.0 (2)	15
11	7	6.3	6.2 (6)	17
12	7	7.3	6.7 (6)	20
13	8	7.3	7.8 (6)	17
14	8	8.3	7.0 (4)	22
15	7	6.3	6.0 (6)	21

*n = number of tests

Streptococcus and *Enterococcus* harmlessly colonise the human pharynx, vagina, gastrointestinal tract and skin, their ability to be involved in infection is well established. Gram positive cocci arranged in chains were first observed in wounds by Billroth during the nineteenth century and the clinical significance of even low numbers of beta haemolytic streptococci in wounds was recognised by military surgeons during World War I (Hepburn, 1919). Their presence in leg ulcers (Schraibman, 1987) and their destructive action on skin grafts applied to burns or ulcers has long been understood by plastic surgeons (Jackson, Lowbury and Topley, 1951) and it is accepted that there is a need to eliminate them before skin grafting (Schraibman, 1990). The clinical significance of group G *Streptococcus* species in chronic leg ulcers has been demonstrated (Cooper, Baragwanath, Hogg and Harding, 1995). More recently honey has been reported to be an effective treatment of Fournier's gangrene (Tahmaz, Erdemir, Kibar, Cosar and Yalcyn, 2006). Streptococci have been implicated in necrotising fasciitis, of which Fournier's gangrene is one form.

MIC values obtained by three test methods indicate that 15 cultures of streptococci isolated from wounds demonstrated susceptibility to concentrations of manuka honey at or below 10% (v/v). Since licensed wound care products containing manuka honey normally contain at least 80% (v/v) of a medical grade honey, there is confidence that streptococci will be inhibited by the concentrations that are likely to be attained in clinical practice by topical application to wounds. Therefore manuka honey seems to offer potential as a wound antiseptic.

Honey is known for its broad spectrum of antimicrobial activity (Molan, 1992). This study demonstrates that streptococci and enterococci were equally susceptible to a medical grade manuka honey *in vitro*. Together with data previously reported for staphylococci (Cooper, Molan and Harding, 2002; French, Cooper and Molan 2005; George and Cutting, 2007; Blair *et al* 2009), coliforms (George and Cutting, 2007; Blair *et al* 2009) and pseudomonads (Cooper, Halas and Molan 2002; George and Cutting, 2007; Blair *et al* 2009), the potential of manuka honey for topical application to wounds colonised with antibiotic-resistant bacterial pathogens is evident. Increased clinical use could impact not only on controlling wound infections but in preventing the spread of infective agents within a wide range of healthcare establishments.

Testing the susceptibility of a test organism to an antimicrobial agent in the laboratory depends on contact time, concentration, temperature, pH, the number and type of test organism and the presence of interfering substances. Guidelines have been established to evaluate the efficacy of antibiotics or antiseptics in laboratory tests. In testing microbial susceptibility it is usual to prepare a serial doubling dilution (where the concentration of inhibitor is halved at each step). In this study a dilution series that varied by intervals of 1% (v/v) honey concentration at each step was used to obtain a more accurate endpoint. Traditionally Kahn tubes were used to

determine MICs. Whereas the growth of test organisms may be easily seen in tubes after the incubation period, growth in the wells in a microtitre plate are more difficult to visualise and a plate reader is often used to deduce the MIC. Because Kahn tubes require larger volumes of microbiological media than wells in microtitre plates and tubes take more time to set up, microtitre plates are often preferred. Until this study susceptibility testing with honey seems to have utilised either broth dilution or agar incorporation methods. It is important to establish that macro-dilution, micro-dilution and agar incorporation methods yield similar endpoints when one honey sample is tested on the same cohort of test organisms.

Conclusion

Three methods (macro-dilution in broth, micro- dilution in broth and agar incorporation) were used to evaluate the susceptibility of 15 cultures of streptococci isolated from wounds to manuka honey. A concentration of 10% (v/v) manuka honey was found to inhibit all of the cultures and all methods gave similar endpoints. However, the efficacy of manuka honey in eradicating streptococci from wounds needs to be evaluated *in vivo*.

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