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Synergy between oxacillin and manuka honey sensitizes methicillin-resistant *Staphylococcus aureus* to oxacillin

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Objectives: Honey is an ancient wound remedy that has recently been introduced into modern clinical practice in developed countries. Manuka honey inhibits growth of methicillin-resistant *Staphylococcus aureus* (MRSA) by preventing cell division. In Gram-negative bacteria a synergistic interaction between honey and antibiotics has been suggested. We aimed to determine the effect of manuka honey on oxacillin resistance in MRSA.

Methods: Inhibition of MRSA by manuka honey and oxacillin separately and in combination was tested by disc diffusion, Etest strips, serial broth dilution, chequerboards and growth curves.

Results: Manuka honey and oxacillin interacted synergistically to inhibit MRSA. Manuka honey reversed oxacillin resistance in MRSA, and down-regulation of *mecR1* was found in cells treated with manuka honey.

Conclusions: Microarray analysis showed that exposure of MRSA to inhibitory concentrations of manuka honey resulted in down-regulation of *mecR1*. Here we demonstrated that subinhibitory concentrations of honey in combination with oxacillin restored oxacillin susceptibility to MRSA. Other honey and antibiotic combinations must now be evaluated.

Keywords: MRSA, MecR1, honey

Introduction

Since its emergence in 1961, methicillin-resistant *Staphylococcus aureus* (MRSA) has become a significant burden on public health globally. Epidemic strains EMRSA-15 and EMRSA-16 (NCTC 13142 and NCTC 13143, respectively) have been most commonly associated with bacteraemia in the UK.[1] With the low number of antimicrobial agents under development, innovative alternatives must be found.

Re-examination of ancient remedies such as garlic, green tea and honey has generated optimism about finding inhibitors for antibiotic-resistant pathogens. Honey has been used for millennia as a topical treatment for wounds and modern wound dressings containing honey are now available on formularies in many developed countries. Registered products include medical grade honey in tubes, ointments, gels, impregnated onto non-adherent dressings or alginate, and non-sticky flexible honey sheets. All are sterilized by gamma irradiation. Many products contain manuka honey produced by bees foraging on manuka bushes in New Zealand. Manuka honey has been shown to eradicate MRSA from colonized wounds and to inhibit MRSA *in vitro* by interrupting cell division.[2] Although β-lactams are not effective against MRSA, combinations of tea extracts and β-lactams have been

demonstrated to restore methicillin susceptibility.[3] Methicillin resistance in MRSA is conferred by the *mec* gene complex, where *mecA* is regulated by MecR1 and MecI; blocking the *mecR1/blaR1* pathway restored antibiotic susceptibility in MRSA.[4] The aim of this study was to investigate whether combinations of manuka honey and oxacillin acted synergistically to increase susceptibility of MRSA to oxacillin.

Materials and methods

EMRSA-15 NCTC 13142 was used throughout this study. Oxacillin susceptibility was determined by antibiotic susceptibility testing (AST) according to the BSAC guidelines, except that Mueller–Hinton agar (MHA) (Oxoid, Cambridge, UK) was used with 5 μg oxacillin discs (Oxoid, Cambridge, UK).[5] The MIC of oxacillin (Sigma, Dorset, UK) was determined by serial doubling dilution in Mueller–Hinton broth (MHB) (Oxoid, Cambridge, UK) in microtitre plates and with oxacillin Etest strips (bioMérieux) on MHA.

The MIC of sterile manuka honey, which was free from antibiotics, was determined in microtitre plates by dilution in MHB as above, except that dilutions varying by 1% (w/v) intervals were used instead of a doubling dilution series.

To identify interaction between oxacillin and manuka honey, oxacillin susceptibility of MRSA was determined by AST and by Etest strips as

Table 1. Susceptibility of MRSA to oxacillin and manuka honey

| | MIC oxacillin | | | |
	medium with no addition (mg/L)	medium containing 5% (w/v) manuka honey (mg/L)	MIC manuka honey (mg/L)	FIC
Etest strip	64	0.075	60 000	0.001 + 0.00000125 = 0.001
Broth dilution	64	0.06	60 000	0.001 + 0.0000001 = 0.001
Chequerboards	64	0.25	60 000	0.001 + 0.000004 = 0.001
Time–kill curves	64	0.25	60 000	0.001 + 0.000004 = 0.001

Assays were performed on at least three occasions and no variation in endpoints was found.

described above with MHA containing either 2.5% or 5% (w/v) manuka honey (subinhibitory concentrations). Similarly, the effect of including 5% (w/v) manuka honey in MHB on the MIC of oxacillin against MRSA was determined in microtitre plates. To investigate synergistic interaction between oxacillin and manuka honey against MRSA, a chequerboard was set up in microtitre plates as previously described; doubling dilutions of oxacillin (256–0.125 mg/L) were dispensed into successive rows and stepwise dilutions of manuka honey varying by 1% (w/v) intervals in successive columns.[6] The fractional inhibition concentration index (FICI) was calculated for each combination using the following formula:

$$FICI = FIC \text{ of oxacillin} + FIC \text{ of manuka honey}$$

where FIC of oxacillin is the MIC of oxacillin in combination/MIC of oxacillin alone, and FIC of manuka honey is the MIC of manuka honey in combination/MIC of manuka honey alone. The results were interpreted as follows: ≥ 0.5, synergy; >0.5 to ≥ 4, additivity; and >4, antagonism.[7]

Time–kill curves for EMRSA-15 were performed using MHB with varying concentrations of oxacillin and manuka honey in microtitre plates incubated at 37°C in a Tecan Infinite plate reader. Optical density was monitored at 550 nm at hourly intervals for 24 h. Growth of MRSA was also monitored with subinhibitory combinations of oxacillin and manuka honey in MHB.

Microarray analysis was performed on RNA extracted from cultures of EMRSA-15 grown in MHB with and without 10% (w/v) honey for four hours. RNA was isolated using an SV Total RNA isolation kit (Promega) and cDNA prepared using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturers' instructions. RNA was then processed, hybridized, stained and scanned on Affymetrix arrays according to the manufacturers' instructions for prokaryotic target preparation.

All experiments were done with three biological replicates and mean values are presented here. The fold changes are corrected and normalized to account for background noise.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (R. E. Jenkins and R. A. Cooper, 2011) and are accessible through GEO Series accession number GSE31592 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31592; reviewer link at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lxatrsyakqwuolc&acc=GSE31592).

Results

Resistance of MRSA to oxacillin was confirmed by AST (where zones of inhibition were not seen) and by Etest strips and broth dilution, where the MIC of oxacillin was found to be 64 mg/L (Table 1). The MIC of manuka honey against MRSA determined
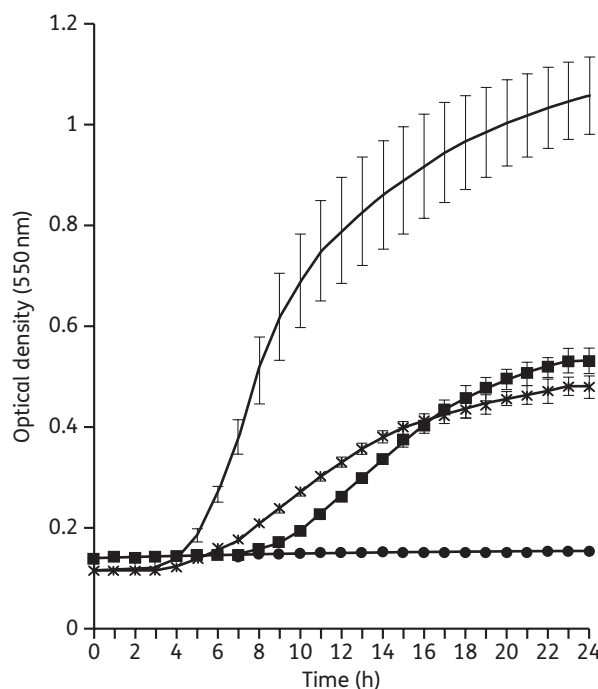

Figure 1. Growth curves of MRSA in MHB. MRSA grown in MHB alone (line with no symbols), MRSA in MHB containing 0.5 mg/L oxacillin (asterisks), MRSA in MHB containing 5% (w/v) manuka honey (filled squares) and MRSA in MHB containing 5% (w/v) manuka honey and 0.25 mg/L oxacillin (filled circles). Vertical bars indicate standard deviation.

by broth dilution was 6% (w/v) or 60 000 mg/L. When subinhibitory concentrations of manuka honey were incorporated into MHA to investigate effects on oxacillin susceptibility of MRSA by AST, inhibition zones of 32 mm diameter around 5 μg oxacillin discs were observed with 5% (w/v) manuka honey. This reversal of oxacillin resistance in MRSA by manuka honey was also observed by testing combinations of oxacillin and manuka honey using Etest strips (Table 1), broth dilutions and chequerboards (Table 1).

Time–kill curves showed that growth of MRSA was prevented by each of 64 mg/L oxacillin or 6% (w/v) manuka honey in MHB (data not shown), but not with 0.5 mg/L oxacillin or 5% (w/v) manuka honey (Figure 1). However, growth of MRSA was prevented when MHB containing 0.25 mg/L oxacillin and 5% (w/v)

manuka honey in combination was used (Figure 1). FICI values were <0.5 and indicated that oxacillin and manuka honey in combination act synergistically to inhibit MRSA at concentrations below the individual MIC values (Table 1).

Microarray analysis showed that the *mecR1* gene product was down-regulated by a factor of 3 in MRSA treated with 10% (w/v) manuka honey for 4 h.

Discussion

Synergy between honey and antibiotics has been investigated previously, but unconvincing data were collected, FICI values were not calculated and mechanisms were not suggested.[8] Our findings indicate that sub-lethal concentrations of manuka honey have a marked effect in enhancing the susceptibility of MRSA to oxacillin. As honey can be used undiluted in dressing wounds, 6% is an easily achievable concentration. The down-regulation of *mecR1* might explain our observations. Methicillin resistance in MRSA is conferred by the *mec* gene complex; *mecA* encodes a penicillin-binding protein [penicillin-binding protein 2a (PBP 2a)] with low binding affinity for β-lactam antibiotics. This allows peptidoglycan biosynthesis to continue in the presence of β-lactams. Regulation of *mecA* is via *mecR1*, which codes for a two-component sensor/signal transducer protein, and *mecI*, which codes for a repressor protein.[9] Oxacillin is a β-lactam that has long been used in characterizing antibiotic susceptibility in MRSA and finding synergy between it and honey gives a rationale for testing further combinations. Manuka honey has already been demonstrated not to select for honey-resistant strains, and using antibiotics in combination with honey ought to reduce risks of further antibiotic resistance emerging.[10]

Reversal of oxacillin resistance in MRSA has been reported for extracts of *Camellia sinensis* (green tea), *Salvia miltiorrhiza* (red sage) and *Glycyrrhiza uralensis* (Chinese liquorice), and the prospect of using combinations of phytochemicals and antibiotics or anticancer drugs in conventional medicine has been raised. Another approach to the restoration of susceptibility of MRSA to methicillin has been to use an antisense oligonucleotide to block the *mecR1*-mediated signalling pathway, but this is currently not available commercially.[9]

Manuka honey was re-introduced into modern medicine in 1999. It has been shown to inhibit MRSA effectively *in vitro* and *in vivo*. The findings reported here suggest that *in vitro* this combination could be beneficial. However, *in vivo* work would need to be conducted to determine whether sufficient penetration of honey and antibiotic is likely to occur in clinical conditions such as wound infections or chronic leg ulcers; if so, patients could potentially benefit from these findings. The presence of manuka honey was shown in this study to restore susceptibility of MRSA to oxacillin; molecular analysis indicated that honey also affected the regulation of the *mecR1* gene, possibly accounting for the restored susceptibility seen here.

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