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

Running Title: Sensitising MRSA to oxacillin

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**Synopsis**

**Objectives**: Honey is an ancient wound remedy that has recently been introduced into modern clinical practice in developed countries. Manuka honey inhibits growth of MRSA by preventing cell division. In Gram negatives syngeristic interaction between honey and antibiotics have 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 disk diffusion, E 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 downregulation of mecR1. Here we demonstrate that sub-inhibitory concentrations of honey in combination with oxacillin restored oxacillin susceptibility to MRSA. Other honey and antibiotic combinations must now be evaluated.

**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 of 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 sterilised 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 colonised 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 reverse methicillin susceptibility.3 Methicillin resistance in MRSA is conferred by the *mec* gene complex, where *mec* A is regulated by mecR1 and *mec*I; 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**

Epidemic methicillin resistant *Staphylococcus aureus* EMRSA-15 NCTC 13142 was used throughout this study. Oxacillin susceptibility was determined by antibiotic sensitivity testing (AST) according to the British Society for Antimicrobial Chemotherapy guidelines, except that Mueller-Hinton agar (MHA) (Oxoid, Cambridge, UK) was used with 5 μg oxacillin disks (Oxoid, Cambridge, UK).5 The minimum inhibitory concentration (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 E 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.

Toidentify interaction between oxacillin and manuka honey, oxacillin sensitivity of MRSA was determined by AST and by E strips as described above with MHA containing either 2.5 or 5% (w/v) manuka honey (sub-inhibitory concentrations). Similarly the effect of including 5% (w/v) manuka honey into 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 µg mL) were dispensed into successive rows and stepwise dilutions of manuka honey varying by 1% (w/v) intervals in successive columns.6 Fractional inhibition concentration index (FICI) was calculated for each combination using the following formula:

FICI = FIC of oxacillin + FIC of manuka honey,

where FIC of oxacillin = MIC of oxacillin in combination/MIC of oxacillin alone,

and FIC of manuka honey = 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 of 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 sub-inhibitory 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 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 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 normalised to account for background noise.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Jenkins *et al*., 2011) and are accessible through GEO Series accession number GSE31592 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31592>).

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**Results**

Resistance of MRSA to oxacillin was confirmed by AST (where zones of inhibition were not seen) and by E strips and broth dilution where the MIC was found to be 64 mg/Loxacillin (Table 1). The MIC of manuka honey against MRSA determined by broth dilution was 6% (w/v) or 60,000 mg/L. When sub-inhibitory 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 disks 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 E strips (Table 1), broth dilutions and chequerboards (Table 1).

In time-kill curves, 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 (Fig. 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 (Fig. 1). FICI values were below 0.5 and indicate that oxacillin and manuka honey in combination act synergistically to inhibit MRSA at concentrations below individual MIC values, respectively (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 hours.

**Discussion**

Synergy between honey and antibiotics has been investigated previously, but unconvincing data was collected, Fractional Inhibitory Concentration Index (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 2a (PBP 2a) with low binding affinity for beta lactam antibiotics. This allows peptidoglycan biosynthesis to continue in the presence of beta lactams. Regulation of *mecA* is via *me*c*R1* which codes for a two component sensor/signal transducer protein and *me*c*I* which codes for a repressor protein.9 Oxacillin is a β lactam that has long been used in characterising 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 sinesis*) green tea, *Saliva 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 restore methicillin sensitivity in MRSA 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 are likely to be met in clinical conditions such as wound infections or chronic leg ulcers where, then patients could potentially benefit from these findings. The presence of manuka honey was shown in this study to restore sensitivity to MRSA to oxacillin, molecular analysis indicated that honey also affected the regulation of the MecR1 gene possibly accounting for the restored sensitivity seen here.

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**Figure 1: Growth curves of MRSA in Mueller-Hinton broth.**

MRSA grown in Mueller Hinton Broth alone (continuous line), MRSA in Mueller Hinton Broth containing 0.5 mg/L oxacillin (\*), MRSA in Mueller Hinton Broth containing 5%(w/v) manuka honey ( ) and MRSA in Mueller Hinton Broth containing 5%(w/v) manuka honey and 0.25 mg/L oxacillin ( ).

**Table 1: Susceptibility of MRSA to oxacillin and manuka honey.**

Assays were performed on at least three occasions and no variation in end-points were found.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **MIC oxacillin** | | **MIC manuka honey**  **(mg/L)** | **Fractional Inhibition Concentration (FIC)** |
| **Media with no addition**  **(mg/L)** | **Media containing 5%(w/v) manuka honey**  **(mg/L)** |
| E 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 |