Article title: Antimicrobial activity of Manuka honey against antibiotic resistant strains of the cell wall free bacteria *Ureaplasma parvum* and *Ureaplasma urealyticum*.

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Running title: Activity of honey against *Ureaplasma*

Significance and impact of the study

Manuka honey is known to have a broad spectrum of antimicrobial activity, with the bacterial cell wall being suggested as a predominant site of action. This study has demonstrated that Manuka honey has activity against *Ureaplasma* spp., a genus of cell-wall free bacteria which are intrinsically resistant to many available antibiotics making treatment inherently difficult. This is the first report of the antimicrobial activity of Manuka honey against a bacterial pathogen, in the absence of a cell well and opens scope for the use of components of Manuka honey as a therapeutic among *Ureaplasma* infections.

Abstract

The susceptibility of the cell-wall free bacterial pathogens *Ureaplasma* spp. to Manuka honey was examined. The minimum inhibitory concentration (MIC) of Manuka honey for four *Ureaplasma urealyticum* and four *Ureaplasma parvum* isolates was determined. Sensitivity to honey was also compared to clinical isolates with resistance to tetracycline, macrolide and fluoroquinolone antibiotics. Finally step-wise resistance training was utilised in an attempt to induce increased tolerance to honey. The MIC was dependent on the initial bacterial load with 7.5 % and 18.0 % w/v honey required to inhibit *U. urealyticum* at 1 and 10^6 colour changing units (CCU), respectively, and 4.8 % and 15.3 % w/v required to inhibit *U. parvum* at 1 and 10^6 CCU, respectively. MIC values were consistently lower for *U. parvum* compared with *U. urealyticum*. Antimicrobial activity was seen against tetracycline resistant, erythromycin resistant and ciprofloxacin resistant isolates at 10^5 CCU. No resistance to honey was observed with fifty consecutive challenges at increasing

concentrations of honey. This is the first report of the antimicrobial activity of Manuka honey against a cell-wall free bacterial pathogen. The antimicrobial activity was retained against antibiotic resistant strains and it was not possible to generate resistant mutants.

Key Words: Antimicrobials, Microbial structure, Infection, Microbial physiology, Resistance

Introduction

Ureaplasma spp. are a genus of bacteria of clinical relevance strongly linked with preterm birth and subsequent development of neonatal complications such as bronchopulmonary dysplasia, intraventricular haemorrhaging and necrotising enterocolitis (Viscardi, 2014). Additionally these pathogens are becoming recognised in sexual health (Zhang et al., 2014, Ondondo et al., 2010) and immune compromised transplant patients (Bharat et al., 2015). The unique physiology of these organisms results in high levels of intrinsic resistance to many clinically available antibiotics. For example, the absence of a peptidoglycan cell wall renders these organisms resistant to all beta-lactam and glycopeptide antibiotics. Only a limited number of antimicrobial classes are available for treatment including the macrolides, tetracyclines, fluoroquinolones and chloramphenicols. With respect to infection during pregnancy and among preterm neonates these options are further limited due to host toxicity issues. Tetracyclines are associated with deposition in growing teeth and bones whereas systemic administration of chloramphenicol is associated with "Grey baby" syndrome. Further complications arise as a result of isolates harbouring acquired resistance to the limited number of available antibiotics, with exception to chloramphenicol (Beeton et al., 2015, Beeton et al., 2009b). For these reasons alternatives are urgently required.

Manuka honey has been shown to be a promising natural product with potent antimicrobial activity against pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.(Jenkins et al., 2011, Jenkins et al., 2012) Unlike many traditional antibiotics which have a single site of action, honey has been suggested to have multiple antimicrobial components such as hydrogen peroxide, high levels of sugars, and methylglyoxal (Maddocks

and Jenkins, 2013). Due to the multifaceted antimicrobial nature of this product it has been difficult to generate resistance *in vitro* (Cooper et al., 2010).

Here we present data demonstrating the first report of antimicrobial activity of Manuka honey against a cell-wall free bacterial pathogen. Additionally, we show no increase in susceptibility for clinical isolates characterised to have known mechanisms of antibiotic resistance, nor could resistance to honey be induced with repeated challenge of strains with concentrations of Manuka honey just below the MIC with classic *in vitro* step-wise training.

Results and discussion

A total of eight antibiotic susceptible *Ureaplasma* strains were initially examined for baseline susceptibility to Manuka honey using the modified broth microdilution method. For both *U. urealyticum* and *U. parvum* the percentage of Manuka honey required to yield inhibition increased in relation to the increase in initial inoculum (from 7.5% at 1 CCU to 18.0% at 10^6 CCU for *U. urealyticum* and 4.8% at 1 CCU to 15.3% at 10^6 for *U. parvum*) (Table 1). At the Clinical & Laboratory Standards Institute (CLSI) recommended inoculum of 10^4 - 10^5 for testing antimicrobials against *Ureaplasma* spp., the mean MIC for *U. urealyticum* was higher than that of *U. parvum* (13.5 vs 12.7 at 10^4 and 16.7 vs 15.8 at 10^5), but this difference was not statistically significant ($p = 0.49$). Following the establishment of baseline MIC values for Manuka honey against both *U. urealyticum* and *U. parvum*, the activity was then assessed against a small representative collection of antibiotic resistant strains. No increase in MIC was noted for any resistant strain at the recommended 10^4 or 10^5 CCU relative to the matched inoculum for each respective antibiotic susceptible species

(Table 2). The antibiotic susceptible strain HPA5 was serially passaged in sub-inhibitory concentrations of Manuka honey in an attempt to generate honey resistant isolates. After 50 serial passages no elevation in Manuka honey MIC was noted (data not shown).

The purpose of this study was to evaluate the antimicrobial activity of Manuka honey against a panel of clinical and laboratory strains of *Ureaplasma* spp. From this we report the first example of antimicrobial activity of Manuka honey against a cell-wall free bacterial pathogen as well as retention of activity against clinically relevant antibiotic resistant strains. Data available to date on the antimicrobial activity of Manuka honey has been generated in respect to typical bacterial pathogens such as *S. aureus* and *P. aeruginosa* (Jenkins et al., 2011, Camplin and Maddocks, 2014). It has been suggested that one of the primary mechanisms of action of Manuka honey is targeting the cell wall murein hydrolase therefore disrupting cellular division (Jenkins et al., 2011). As a result of reductive evolution ureaplasmas have lost the biosynthetic capabilities to synthesise the peptidoglycan cell wall. From the data presented here we can speculate there are additional cellular targets other than the cell wall which leads to the antimicrobial activity, which reflects that previously suggested by Jenkins *et al*., (Jenkins et al., 2014). In addition non-specific effects as a result of osmotic imbalances may have contributed to the antimicrobial activity. The MIC values for both *Ureaplasma* spp. were lower than those reported for the ATCC 9027 strain of *P. aeruginosa* (25.6 % w/v), yet comparable to a clinical *P. aeruginosa* isolate (15.3 % w/v),(Camplin and Maddocks, 2014) but were much higher than those previously reported for *S. aureus* <6 % w/v (Jenkins et al., 2012). These subtle differences may be due to the sites of action upon the pathogen in question, such as the cell wall in *S. aureus*, or differences in the Unique Manuka Factor between batches of honey

114 examined. When examining the MIC values between the *Ureaplasma* spp. we noted that *U.*
115 *urealyticum* had consistently higher MIC values at the CLSI recommended inoculum of 10^4 to
116 10^5 when compared with *U. parvum*. Although this was not a statistically significant
117 difference, this reflects the observations in species difference seen when examining the
118 activity of antibiotics against these pathogens (Beeton et al., 2016). Of clinical relevance was
119 the observation that bacterial load played a substantial role in the MIC for both *U. parvum*
120 and *U. urealyticum*. Low grade infections would be treatable with much lower
121 concentrations of honey, where as those with high titres, as seen clinically, would require
122 much higher concentrations (Beeton et al., 2016). Antibiotic resistant strains have been
123 reported for the major classes of antibiotics effective against ureaplasmas, most notably the
124 macrolides, tetracyclines and fluoroquinolones (Beeton et al., 2009b, Beeton et al., 2015).
125 For this reason we examined the antimicrobial activity of honey against a panel of antibiotic
126 resistant clinical isolates. We observed retention of antimicrobial activity against these
127 isolates suggesting no cross-resistance from either antibiotic resistance mechanism or the
128 activity of honey. This is of significance in the case of preterm neonatal infections where
129 macrolides are regarded the predominant antibiotic class of choice. Pereyre *et al*. 2007,
130 have previously demonstrated the ease by which ureaplasmas can acquire point mutations
131 resulting in the development of resistance following exposure to macrolides via step wise
132 resistance training (Pereyre et al., 2007). Similarly resistance to fluoroquinolones among
133 *Ureaplasma* spp. results from the accumulation of mutations in the quinolone resistance
134 determining regions (Beeton et al., 2009a). The data presented here demonstrated that it
135 was not possible to generate isolates with an increased honey MIC following a similar time
136 frame in which macrolide resistance was generated (Pereyre et al., 2007). This is likely due
137 to the suggested multiple antimicrobial agents present with in Manuka honey (Maddocks

and Jenkins, 2013). The inability to generate mutants is in line with previous reports for *S. aureus* and *P. aeruginosa* although a report by Camplin and Maddocks demonstrated an increase in MIC for *P. aeruginosa* isolates recovered from honey treated *in vitro* biofilms (Cooper et al., 2010, Camplin and Maddocks, 2014).

In summary we have successfully demonstrated antimicrobial activity of Manuka honey against a bacterial pathogen with high levels of intrinsic and acquired antibiotic resistance in the absence of a cell wall. The mechanisms by which Manuka honey exerts antimicrobial activity in this atypical bacterial pathogen of increasing clinical significance warrants further investigation.

Materials and methods

A total of eight antibiotic susceptible *Ureaplasma* strains were examined. These comprised of four *U. urealyticum* including two clinical isolates (HPA99 and W11) and two reference strains (ATCC 27814 SV2 and ATCC 27618 SV8), in addition four *U. parvum* including two clinical isolates (HPA2 and HPA5) and two reference strains (ATCC 700970 SV3 and ATCC 27818 SV6). Representative antibiotic resistant strains ATCC 33175 SV9 (tetracycline resistant), UHWO10 (erythromycin resistant) and HPA116 (ciprofloxacin resistant) were included (Beeton et al., 2009b, Beeton et al., 2015). All *Ureaplasma* isolates were grown in *Ureaplasma* selective media purchased from Mycoplasma Experience (Surrey, UK). Susceptibility to Activon 100% Medical Grade Manuka honey, purchased from Advancis Medical (Nottinghamshire, UK), was determined using CLSI M43-A guidelines for antimicrobial susceptibility testing for human mycoplasmas. In brief, a dilution gradient of

honey prepared in Ureaplasma Selective Media from 20 % w/v to 0 % w/v (2% increments) were prepared. 180 µl of each dilution was then added to all wells with in columns of a 96 well microtiter plate. For example 180 µl 20 % w/v honey was added to wells A12 – H12, 180 µl 18 % w/v honey was added to wells A11 – H11. Finally 20 µl of a logarithmic phase culture of *Ureaplasma* was added to the all wells from A1 – A12. 1:10 dilutions from this were made across the plate from column one though to column eight as a means for determining the inhibitory activity of the Manuka honey at multiple concentrations of bacteria. Plates were sealed with an adhesive sealing film and incubated statically at 37 ^{o}C until all colour change had ceased as determined visually (c.a 48 hours). Colour changing units (CCU) were defined by determining the final dilution in which colour change had occurred, orange to red due to increased pH as a result of urea hydrolysis, therefore giving one CCU. From this it was then possible to work back through the dilution gradient to determine the percentage of honey required to inhibit the growth of *Ureaplasma* at each CCU. The methodology as previously described by Pereyre *et al*., was used to select for honey resistant mutants using the antibiotic susceptible strain HPA5 (Pereyre et al., 2007). Statistical analysis was performed using Minitab version 17.0 to determine the statistical significance using a one-way ANOVA.

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Transparency declarations

None to declare

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	Colour Changing Units (CCU)						
	1	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**
U. urealyticum							
ATCC 27814 SV2	4.0 ± 3.2	7.0 ± 5.5	11.3 ± 1.1	11.3 ± 1.1	12.7 ± 1.1	16.7 ± 4.2	16.0 ± *
HPA99	7.3 ± 4.2	8.7 ± 3.1	9.3 ± 2.3	10.7 ± 1.2	12.7 ± 1.2	17.0 ± 4.2	N/A
W11	8.7 ± 4.2	10.0 ± 3.5	10.0 ± 3.5	12.0 ± 3.5	13.3 ± 3.1	14.0 ± *	20.0 ± *
ATCC 27618 SV8	10.0 ± 2.0	12.0 ± 2.0	14.0 ± 0.0	14.0 ± 0.0	15.3 ± 2.3	19.0 ± 1.4	N/A
U.u mean	7.5 ± 2.6	9.4 ± 2.1	11.1 ± 2.1	12.0 ± 1.4	13.5 ± 1.2	16.7 ± 2.1	18.0 ± 2.8
U. parvum							
HPA5	2.3 ± 1.5	9.3 ± 6.4	11.3 ± 4.6	12.0 ± 3.45	12.7 ± 2.3	16.7 ± 1.2	20.0 ± *
ATCC 700970 SV3	7.3 ± 4.6	10.7 ± 1.2	10.7 ± 1.2	11.3 ± 2.3	12.7 ± 2.3	18.0 ± *	N/A
ATCC 27818 SV6	2.3 ± 1.6	11.3 ± 1.1	12.7 ± 1.2	12.7 ± 1.2	13.3 ± 1.2	15.3 ± 3.0	12.0 ± *
HPA2	7.3 ± 3.0	10.7 ± 1.2	11.3 ± 1.2	11.3 ± 1.1	12.0 ± 0.0	13.3 ± 2.3	14.0 ± 2.8
U.p mean	4.8 ± 2.9	10.5 ± 0.8	11.5 ± 0.8	11.8 ± 0.7	12.7 ± 0.5	15.8 ± 2.0	15.3 ± 4.2

Table 1. Antimicrobial activity of Manuka honey against varying inoculum numbers of *Ureaplasma urealyticum* and *Ureaplasma parvum* isolates. Results represent the mean Manuka honey minimum active dilution (% w/v) as well as standard deviation (triplicates). '*' indicates only a single replicate was tested. CLSI guidelines recommend a level of 10^4 – 10^5 CCU for reliable antimicrobial susceptibility testing. N/A = non-applicable. U.u = *U. urealyticum.* U.p = *U. parvum*

	Colour Changing Units (CCU)						
	1	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**
***Ureaplasma* spp.**							
ATCC 33175 SV9 (Tet^r)	6.7 ± 5.0	9.3 ± 3.0	10.7 ± 2.3	10.7 ± 2.3	11.3 ± 1.2	11.3 ± 1.2	12.0 ± 2.0
UHWO10 (Ery^r)	7.0 ± 5.6	8.0 ± 5.3	8.0 ± 5.3	8.0 ± 5.3	8.7 ± 4.2	9.3 ± 5.0	10.0 ± 5.3
HPA116 (Cip^r)	8.0 ± 3.6	9.3 ± 4.6	10.0 ± 3.5	10.7 ± 4.2	11.3 ± 4.6	12.0 ± 3.5	12.0 ± 3.5

Table 2. Antimicrobial activity of Manuka honey against varying inoculum numbers of antibiotic resistant *Ureaplasma spp*.

Results represent the mean Manuka honey minimum active dilution (% w/v) as well as standard deviation (triplicates). ATCC 33175 SV9 (Tet^r) represents a tetracycline resistant strain, UHWO10 (Ery^r) represents an erythromycin resistant strain and HPA116 (Cip^r) indicates a ciprofloxacin resistant strain. CLSI guidelines recommend a level of 10^4 – 10^5 CCU for reliable antimicrobial susceptibility testing.