1   Article title: Antimicrobial activity of Manuka honey against antibiotic resistant strains of the

2   cell wall free bacteria *Ureaplasma parvum* and *Ureaplasma urealyticum*.

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12   Running title: Activity of honey against *Ureaplasma*

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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

37   concentrations of honey. This is the first report of the antimicrobial activity of Manuka

38   honey against a cell-wall free bacterial pathogen. The antimicrobial activity was retained

39   against antibiotic resistant strains and it was not possible to generate resistant mutants.

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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

67 and Jenkins, 2013). Due to the multifaceted antimicrobial nature of this product it has been

68 difficult to generate resistance *in vitro* (Cooper et al., 2010).

69

70 Here we present data demonstrating the first report of antimicrobial activity of Manuka

71 honey against a cell-wall free bacterial pathogen. Additionally, we show no increase in

72 susceptibility for clinical isolates characterised to have known mechanisms of antibiotic

73 resistance, nor could resistance to honey be induced with repeated challenge of strains with

74 concentrations of Manuka honey just below the MIC with classic *in vitro* step-wise training.

75

Results and discussion

77 A total of eight antibiotic susceptible *Ureaplasma* strains were initially examined for

78 baseline susceptibility to Manuka honey using the modified broth microdilution method. For

79 both *U. urealyticum* and *U. parvum* the percentage of Manuka honey required to yield

80 inhibition increased in relation to the increase in initial inoculum (from 7.5% at 1 CCU to

81 18.0% at 10^6 CCU for *U. urealyticum* and 4.8% at 1 CCU to 15.3% at 10^6 for *U. parvum*)

82 (Table 1). At the Clinical & Laboratory Standards Institute (CLSI) recommended inoculum of

83 10^4 - 10^5 for testing antimicrobials against *Ureaplasma* spp., the mean MIC for *U.*

84 *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),

85 but this difference was not statistically significant (p = 0.49). Following the establishment of

86 baseline MIC values for Manuka honey against both *U. urealyticum* and *U. parvum*, the

87 activity was then assessed against a small representative collection of antibiotic resistant

88 strains. No increase in MIC was noted for any resistant strain at the recommended 10^4 or

89 10^5 CCU relative to the matched inoculum for each respective antibiotic susceptible species

5

90   (Table 2). The antibiotic susceptible strain HPA5 was serially passaged in sub-inhibitory

91   concentrations of Manuka honey in an attempt to generate honey resistant isolates. After

92   50 serial passages no elevation in Manuka honey MIC was noted (data not shown).

93

94   The purpose of this study was to evaluate the antimicrobial activity of Manuka honey

95   against a panel of clinical and laboratory strains of *Ureaplasma* spp. From this we report the

96   first example of antimicrobial activity of Manuka honey against a cell-wall free bacterial

97   pathogen as well as retention of activity against clinically relevant antibiotic resistant

98   strains. Data available to date on the antimicrobial activity of Manuka honey has been

99   generated in respect to typical bacterial pathogens such as *S. aureus* and *P. aeruginosa*

100 (Jenkins et al., 2011, Camplin and Maddocks, 2014). It has been suggested that one of the

101 primary mechanisms of action of Manuka honey is targeting the cell wall murein hydrolase

102 therefore disrupting cellular division (Jenkins et al., 2011).   As a result of reductive

103 evolution ureaplasmas have lost the biosynthetic capabilities to synthesise the

104 peptidoglycan cell wall. From the data presented here we can speculate there are

105 additional cellular targets other than the cell wall which leads to the antimicrobial activity,

106 which reflects that previously suggested by Jenkins *et al*., (Jenkins et al., 2014). In addition

107 non-specific effects as a result of osmotic imbalances may have contributed to the

108 antimicrobial activity. The MIC values for both *Ureaplasma* spp. were lower than those

109 reported for the ATCC 9027 strain of *P. aeruginosa* (25.6 % w/v), yet comparable to a clinical

110 *P. aeruginosa* isolate (15.3 % w/v),(Camplin and Maddocks, 2014) but were much higher

111 than those previously reported for *S. aureus* <6 % w/v (Jenkins et al., 2012). These subtle

112 differences may be due to the sites of action upon the pathogen in question, such as the cell

113 wall in *S. aureus*, or differences in the Unique Manuka Factor between batches of honey

6

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

138 and Jenkins, 2013). The inability to generate mutants is in line with previous reports for *S.*

139 *aureus* and *P. aeruginosa* although a report by Camplin and Maddocks demonstrated an

140 increase in MIC for *P. aeruginosa* isolates recovered from honey treated *in vitro* biofilms

141 (Cooper et al., 2010, Camplin and Maddocks, 2014).

142

143 In summary we have successfully demonstrated antimicrobial activity of Manuka honey

144 against a bacterial pathogen with high levels of intrinsic and acquired antibiotic resistance in

145 the absence of a cell wall. The mechanisms by which Manuka honey exerts antimicrobial

146 activity in this atypical bacterial pathogen of increasing clinical significance warrants further

147 investigation.

148

149 ## Materials and methods

150 A total of eight antibiotic susceptible *Ureaplasma* strains were examined. These comprised

151 of four *U. urealyticum* including two clinical isolates (HPA99 and W11) and two reference

152 strains (ATCC 27814 SV2 and ATCC 27618 SV8), in addition four *U. parvum* including two

153 clinical isolates (HPA2 and HPA5) and two reference strains (ATCC 700970 SV3 and ATCC

154 27818 SV6). Representative antibiotic resistant strains ATCC 33175 SV9 (tetracycline

155 resistant), UHWO10 (erythromycin resistant) and HPA116 (ciprofloxacin resistant) were

156 included (Beeton et al., 2009b, Beeton et al., 2015). All *Ureaplasma* isolates were grown in

157 *Ureaplasma* selective media purchased from Mycoplasma Experience (Surrey, UK).

158 Susceptibility to Activon 100% Medical Grade Manuka honey, purchased from Advancis

159 Medical (Nottinghamshire, UK), was determined using CLSI M43-A guidelines for

160 antimicrobial susceptibility testing for human mycoplasmas. In brief, a dilution gradient of

161 honey prepared in Ureaplasma Selective Media from 20 % w/v to 0 % w/v (2% increments)

162 were prepared. 180 µl of each dilution was then added to all wells with in columns of a 96

163 well microtiter plate. For example 180 µl 20 % w/v honey was added to wells A12 – H12, 180

164 µl 18 % w/v honey was added to wells A11 – H11. Finally 20 µl of a logarithmic phase

165 culture of *Ureaplasma* was added to the all wells from A1 – A12. 1:10 dilutions from this

166 were made across the plate from column one though to column eight as a means for

167 determining the inhibitory activity of the Manuka honey at multiple concentrations of

168 bacteria. Plates were sealed with an adhesive sealing film and incubated statically at 37 $^{\circ}$C

169 until all colour change had ceased as determined visually (c.a 48 hours). Colour changing

170 units (CCU) were defined by determining the final dilution in which colour change had

171 occurred, orange to red due to increased pH as a result of urea hydrolysis, therefore giving

172 one CCU. From this it was then possible to work back through the dilution gradient to

173 determine the percentage of honey required to inhibit the growth of *Ureaplasma* at each

174 CCU. The methodology as previously described by Pereyre *et al*., was used to select for

175 honey resistant mutants using the antibiotic susceptible strain HPA5 (Pereyre et al., 2007).

176 Statistical analysis was performed using Minitab version 17.0 to determine the statistical

177 significance using a one-way ANOVA.

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Acknowledgments

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184 Transparency declarations

185 None to declare

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187 References

188

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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

240

241 **Table 1. Antimicrobial activity of Manuka honey against varying inoculum numbers of *Ureaplasma urealyticum* and**

242 ***Ureaplasma parvum* isolates.** Results represent the mean Manuka honey minimum active dilution (% w/v) as well as standard

243 deviation (triplicates). '*' indicates only a single replicate was tested. CLSI guidelines recommend a level of 10^4 – 10^5 CCU for

244 reliable antimicrobial susceptibility testing. N/A = non-applicable. U.u = *U. urealyticum.* U.p = *U. parvum*

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	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

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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.

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