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

3

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11

12 Running title: Activity of honey against *Ureaplasma*

13

14

15 Significance and impact of the study

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

23

24 Abstract

25 The susceptibility of the cell-wall free bacterial pathogens *Ureaplasma* spp. to Manuka
26 honey was examined. The minimum inhibitory concentration (MIC) of Manuka honey for
27 four *Ureaplasma urealyticum* and four *Ureaplasma parvum* isolates was determined.
28 Sensitivity to honey was also compared to clinical isolates with resistance to tetracycline,
29 macrolide and fluoroquinolone antibiotics. Finally step-wise resistance training was utilised
30 in an attempt to induce increased tolerance to honey. The MIC was dependent on the initial
31 bacterial load with 7.5 % and 18.0 % w/v honey required to inhibit *U. urealyticum* at 1 and
32 10^6 colour changing units (CCU), respectively, and 4.8 % and 15.3 % w/v required to inhibit
33 *U. parvum* at 1 and 10^6 CCU, respectively. MIC values were consistently lower for *U. parvum*
34 compared with *U. urealyticum*. Antimicrobial activity was seen against tetracycline
35 resistant, erythromycin resistant and ciprofloxacin resistant isolates at 10^5 CCU. No
36 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.

40

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

42

43

44 Introduction

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

61

62 Manuka honey has been shown to be a promising natural product with potent antimicrobial
63 activity against pathogens such as *Staphylococcus aureus* and *Pseudomonas*
64 *aeruginosa*.(Jenkins et al., 2011, Jenkins et al., 2012) Unlike many traditional antibiotics
65 which have a single site of action, honey has been suggested to have multiple antimicrobial
66 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

76 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 10⁶ CCU for *U. urealyticum* and 4.8% at 1 CCU to 15.3% at 10⁶ for *U. parvum*)
82 (Table 1). At the Clinical & Laboratory Standards Institute (CLSI) recommended inoculum of
83 10⁴ - 10⁵ 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 10⁴ and 16.7 vs 15.8 at 10⁵),
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 10⁴ or
89 10⁵ CCU relative to the matched inoculum for each respective antibiotic susceptible species

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

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 °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.

178

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184 Transparency declarations

185 None to declare

186

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188

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238

239

	Colour Changing Units (CCU)						
	1	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶
<i>U. urealyticum</i>							
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
<i>U. parvum</i>							
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 10⁴ – 10⁵ CCU for

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

245

246

247

248

	Colour Changing Units (CCU)						
	1	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶
<i>Ureaplasma spp.</i>							
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

249

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

251 Results represent the mean Manuka honey minimum active dilution (% w/v) as well as standard deviation (triplicates). ATCC 33175

252 SV9 (Tet^r) represents a tetracycline resistant strain, UHWO10 (Ery^r) represents an erythromycin resistant strain and HPA116 (Cip^r)

253 indicates a ciprofloxacin resistant strain. CLSI guidelines recommend a level of 10⁴ – 10⁵ CCU for reliable antimicrobial

254 susceptibility testing.

255