Susceptibility of Helicobacter pylori to the antibacterial activity of manuka honey

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Summary
Honey is a traditional remedy for dyspepsia, and is still used for this by some medical practitioners although there is no rational basis for its use. The finding that Helicobacter pylori is probably the causative agent in many cases of dyspepsia has raised the possibility that the therapeutic action of honey may be due to its antibacterial properties. Consequently, the sensitivity of Helicobacter pylori to honey was tested, using isolates from biopsies of gastric ulcers. It was found that all five isolates tested were sensitive to a 20% (v/v) solution of manuka honey in an agar well diffusion assay, but none showed sensitivity to a 40% solution of a honey in which the antibacterial activity was due primarily to its content of hydrogen peroxide. Assessment of the minimum inhibitory concentration by inclusion of manuka honey in the agar showed that all seven isolates tested had visible growth over the incubation period of 72 h prevented completely by the presence of 5% (v/v) honey.

Introduction
There have been several reports of upper gastrointestinal dyspepsia, including gastritis, duodenitis and ulceration, being successfully treated with oral dosage of honey1-7. This treatment reported is a return to traditional therapy, honey having been used as a medicine since ancient times8,9. In the work that has been carried out in Islamic countries it is also based on a fundamental belief in the words regarding honey in the Holy Qu‘ran ‘wherein is healing for mankind’10.

These reports are of interest because conventional treatment of gastric and duodenal ulceration is far from satisfactory, as most drugs used suppress but do not cure the ulceration, with slow healing rates and a very high rate of relapse - 80% at 1 year and 100% at 2 years11. Even with maintenance therapy with ranitidine 150 mg at night, the relapse rate is 45%12. Medication for gastric and duodenal ulcers is of high cost: therapy with H2-receptor antagonists is usually maintained for 1 year, and some patients need permanent treatment13.

Treatment with honey is much less expensive and appears to need less time6. However, there is a tendency of some practitioners to prematurely reject ‘alternative medicine’ if it does not have a rational basis. However, the recent finding that gastritis and duodenal ulceration appear to be caused by infection with Helicobacter pylori14,15 raised a testable scientific hypothesis, that the healing effect of honey on gastritis and ulcers occurs through its well established antibacterial activity.

Eradication of Helicobacter pylori gives a long-term cure of duodenal ulcers, preventing the high rate of relapse seen otherwise16-19. Currently a combination of two or three antimicrobials is necessary for a reasonable degree of eradication of Helicobacter pylori, one of these being a bismuth compound17. The antibacterial properties of honey have been established by a large amount of laboratory research, which has recently been comprehensively reviewed20. In many reports honey has been shown to be effective in inhibiting a wide range of bacterial species when diluted to concentrations at which its osmolarity would not be effective. However, there have been no reports of any species of Campylobacter or Helicobacter being tested, so it is not known whether Helicobacter pylori could be expected to be affected by the concentrations of honey that would be achieved in the stomach by the dosage of honey given. Therefore, the present study was undertaken to find the susceptibility of Helicobacter pylori to the antibacterial activity of honey.

The antibacterial activity of honey varies very markedly, and depends on the floral source of the honey21. The major antibacterial factor in most honeys is hydrogen peroxide, produced in the honey by the action of glucose oxidase which is added to the honey by the bee, but some antibacterial activity is due to substances which are derived from the flowers. If honey heals gastritis and ulcers by affecting Helicobacter pylori, it may be the phytochemical content of the honey that is involved rather than the osmolarity or the hydrogen peroxide content of the honey. This was taken into account in the present study.

The occurrence of phytochemical antibacterial factors in honey has been studied in a survey of the antibacterial activity of 345 samples of commercially available New Zealand honey from 26 different floral sources. This was done by testing with catalase added to distinguish between antibacterial activity due to hydrogen peroxide generated in the honey, and activity due to non-peroxide factors21. An agar well diffusion assay with Staphylococcus aureus was used, that was not susceptible to the osmotic effects of the honey. Honey from only one floral source, manuka (Leptospermum scoparium), showed a high level of this type of activity in a significant proportion of the samples, the fairly high antibacterial activity of manuka honey (up to the equivalent of 33% v/v phenol) being in many cases due entirely to this non-peroxide component.
In view of these findings, the present study on the susceptibility of Helicobacter pylori to honey was carried out with both manuka honey and another honey in which the antibacterial activity was due primarily to hydrogen peroxide. The antibacterial activity of both of these honeys was compared with that of an 'artificial honey' formulated to match the content of sugars and gluconic acid in honey, which could possibly also affect the bacterium.

Materials and methods
Selection of honeys
Unpasteurized centrifugally extracted monofloral honeys were used. They were selected as having activity against Staphylococcus aureus that was close to the median values found in the survey of New Zealand honeys to for honeys with activity due to hydrogen peroxide, and for manuka honeys with non-peroxide activity when tested by the same method as used in the survey. The median non-peroxide activity of the 19 manuka honeys with detectable non-peroxide activity in the earlier survey was equivalent to 15.5% (w/v) phenol: the non-peroxide activity of the manuka honey selected for the current study was equivalent to 13.2% (w/v) phenol. The median hydrogen peroxide activity of the 228 honeys with detectable hydrogen peroxide activity in the earlier survey was equivalent to 17.5% (w/v) phenol: the hydrogen peroxide activity of the other honey selected for the current study (from rewarewa, Knightia excelsa) was equivalent to 21.5% (w/v) phenol, and its non-peroxide activity was undetectable (ie <2% phenol).

The honeys were identified by the apiarists supplying them, identification being based on the flavour, colour and aroma of each honey, also the season and location of its production. The identity of the honeys selected was confirmed by pollen analysis.

Homogeneity in the honeys was ensured by thorough agitation at the time of extraction from the combs. The honeys were stored in the dark at 5°C in 201 polyethylene buckets wit tight-fitting lids.

The 'artificial honey' was prepared by adding gluconic acid lactone to distilled water until a pH of 3.9 was achieved, after allowing hydrolysis of the lactone to occur. To 17.5 ml of this solution was added 40.0 g fructose, 36.2 g glucose and 2.8 g sucrose. The solution was heated briefly to 50°C to aid the dissolving of the sugars.

Preparation of honey samples for testing
Solutions of honey for testing were handled aseptically, and were protected from bright light to prevent photodegradation of the glucose oxidase that gives rise to hydrogen peroxide in honey. The honey solutions were prepared just before inoculation to ensure that there was no loss of hydrogen peroxide in the solutions used to test total activity.

Ten grams of honey was weighed out and mixed into 10 ml of distilled water to achieve a 40% (v/v) solution. This solution was sterilized by filtration through a 0.2μm membrane, and was further diluted with sterile distilled water if required.

Culturing of bacteria
Helicobacter pylori were isolated from biopsy samples taken from the margin of gastric ulcers. These samples were processed using a glass tissue grinder, and were plated onto blood agar, chocolate agar and Thayer Martin agar. Also from each processed sample a slope of Christensen urea medium was inoculated and a slide prepared. The plates and urea slope were incubated at 37°C in a microaerophilic atmosphere for 7 days.

The slide preparation was stained using 1% acridine orange and examined for the presence of curved or S-shaped bacilli. We have found that Helicobacter pylori is much easier to detect with acridine orange stain than with Gram stain. After 7 days' incubation, colonies were observed that were 1-2 mm in diameter, dome shaped, glistening and had no apparent pigment. Gram stain of the colony showed characteristic curved, Gram negative bacilli and they exhibited a rapid strong urease reaction.

Cultures are easily contaminated owing to the long incubation in humid atmospheric conditions, therefore Thayer Martin agar was used throughout the subsequent study because of its selectivity.

Preliminary screening of susceptibility of bacteria to honey
An agar well diffusion assay was used to determine if Helicobacter pylori was susceptible to the antibacterial activity of the various types of honey. Using a 1μl standard loop, a colony of each isolate of Helicobacter pylori was picked from a plate and a confluent lawn spread onto a fresh plate. Wells were cut in the agar with a cooled flame and a solution of hydrogen peroxide added to the well. The wells in each plate were then filled with 150 μL of 20% (v/v) and 40% (v/v) solutions of the 'artificial honey', manuka honey and the other honey. The plates were incubated at 37°C in a microaerophilic atmosphere and observed daily.

Determination of minimum inhibitory concentration of honey
Plates were prepared from Thayer Martin agar mixed just before pouring with an appropriate volume of 40% (v/v) solution of manuka honey to achieve a range of plates containing manuka honey at concentrations from 2.5-10% (v/v). Control plates were prepared with no honey added. Using a colorimeter, suspensions of Helicobacter pylori isolates were made in 0.45% saline, equivalent to a 0.5 McFarland standard (approximately

<table>
<thead>
<tr>
<th>Isolates of H pylori</th>
<th>Manuka honey</th>
<th>40% (v/v)</th>
<th>20% (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean zones of inhibition</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(diameter, mm ± SD)*</td>
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<td></td>
<td></td>
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<tr>
<td>After 48 h incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>22.3 (±1.2)</td>
<td>17.5 (±3.5)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>23.7 (±1.5)</td>
<td>16.3 (±1.5)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>22.3 (±2.1)</td>
<td>15.0 (±1.0)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>23.0 (±0.0)</td>
<td>17.0 (±1.0)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>25.6 (±0.6)</td>
<td>21.5 (±2.1)</td>
<td></td>
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<tr>
<td>After 96 h incubation</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>A</td>
<td>17.7 (±0.6)</td>
<td>0</td>
<td></td>
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<tr>
<td>B</td>
<td>20.7 (±1.5)</td>
<td>14.0 (±1.7)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>18.3 (±2.1)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>16.0 (±0.0)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>25.0 (±0.0)</td>
<td>19.5 (0.7)</td>
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</tr>
</tbody>
</table>

*Each isolate was tested in triplicate
Table 2. Determination of the minimum inhibitory concentration of manuka honey against seven isolates of Helicobacter pylori. The results are shown as growth observed after 72 h incubation of the isolates on plates with various concentrations of honey incorporated in the agar. Duplicate plates were set up for all determinations.

<table>
<thead>
<tr>
<th>Isolates of</th>
<th>Honey</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori</td>
<td>10% (w/w)</td>
<td>5% (w/w)</td>
</tr>
<tr>
<td>A</td>
<td>-/−</td>
<td>-/−</td>
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<td>B</td>
<td>-/−</td>
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<td>C</td>
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<td>F</td>
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<td>-/−</td>
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<tr>
<td>G</td>
<td>-/−</td>
<td>-/−</td>
</tr>
</tbody>
</table>

+= Good growth; w= weak growth; − = no growth

10⁶ CFU/ml. A confluent lawn of each isolate of Helicobacter pylori was spread onto each plate as in the preliminary screening. The plates were incubated at 37°C in a microaerophilic atmosphere and observed after 72 h.

Results
In the initial screening with the agar diffusion assay only the manuka honey showed any sign of inhibition of growth of Helicobacter pylori. The diameter of the clear zones around the wells was measured, and the results are shown in Table 1. It can be seen that manuka honey has a good antibacterial activity against all five isolates even when diluted to 20%, but some of the isolates were able to grow at this lower concentration after prolonged incubation allowed the antibacterial activity to become lowered by diffusion.

The results of the determination of the minimum inhibitory concentration of manuka honey against seven isolates of Helicobacter pylori are shown in Table 2. It can be seen that complete inhibition of growth of all seven isolates tested, over an incubation period of 72 h, was achieved by the presence of manuka honey at 5% of its full strength; partial inhibition of some isolates was achieved at 2.5%.

Discussion
This study has shown that Helicobacter pylori can be inhibited by the antibacterial activity of honey at concentrations that could be achieved in the stomach by reasonable oral dosage. However, the results obtained with the agar diffusion assay of honey at higher concentrations clearly show that the hydrogen peroxide generated in honey and the osmolarity are of no importance. Although there can be problems in comparing different types of antibacterial substances with this type of assay, others have found that the hydrogen peroxide activity in honey is detected more readily than the non-peroxide activity by an agar well diffusion assay. Helicobacter pylori is known to produce catalase, which would account for its resistance to the honey with the high level of hydrogen peroxide. It cannot be said that Helicobacter pylori is not susceptible to the hydrogen peroxide activity in higher concentrations of honey, or to the osmotic activity, because in the assay the concentration in the agar will decrease by diffusion and may subsequently allow growth. This would be particularly so in the case of such a slow growing species as Helicobacter pylori. However, with the different types of activity being compared under the same conditions it can clearly be seen that the activity of manuka honey has far more effect on the species than does the osmolarity or hydrogen peroxide content of honey.

In order to determine the minimum inhibitory concentration of manuka honey it was necessary to test with the honey in the growth medium rather than use an agar diffusion assay, because with diffusion occurring from a well in the agar the effective concentration would not be known. Unless the antibacterial component of manuka honey is prevented from reaching Helicobacter pylori at the site of infection, this would be the concentration that would have to be achieved in the gastric fluid by oral dosage of honey. It could be achieved with a dose of 2.5 ml of honey entering the 50 ml or less of gastric fluid that would be expected to be present in the stomach before a meal. A higher dosage would be required if there are diffusion barriers involved in reaching the site of infection. Although the antibacterial component of manuka honey has not been fully characterized, it is known to be very hydrophilic and to have a molecular weight of about 500 Da, suggesting that it should diffuse readily. It is also known to be stable at pH 12.6.

Rauws and Tytgat in their monograph on Helicobacter pylori have expressed the opinion that there is 'a great need for further improvement of the actual therapeutic regimens to eradicate C. pylori'. Currently two or three antimicrobials are necessary for a reasonable degree of eradication as there is no single drug to combat Helicobacter pylori. This makes it unsuitable for most patients. Many drugs, and especially antibiotics have serious side-effects, like drug hypersensitivity, antibiotic-associated colitis, and antibiotic resistance. There is also the risk of nephrotoxicity when bismuth is used yet so far bismuth seems to be an essential element in the therapeutic schemes.

Honey is a very bland treatment, and in fact can protect the stomach from the damaging action of other substances. However, although manuka honey shows potential for use as a low-cost innocuous agent against Helicobacter pylori, its usefulness clinically is not known. Helicobacter pylori is susceptible to many antibacterial agents in vitro but only a few are effective in vivo. A clinical trial would be necessary to determine if manuka honey is effective in the treatment of dyspepsia in which Helicobacter pylori is known to be involved.

The results reported here also raise the possibility that an antibacterial agent useful against Helicobacter pylori may be found in the pollen or an extract of the manuka tree. Further laboratory work would show if this were so, but toxicity testing would be necessary before a clinical trial of such an agent could be conducted. Manuka honey, a common floral type in New Zealand, has been ingested in large quantities by a large number of people for a long time without any adverse effects coming to light.

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