Comprehensive In Situ Killing of Six Common Wound Pathogens With Manuka Honey Dressings Using a Modified AATCC-TM100

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Abstract: Objective. While Manuka honey in vitro is strongly antimicrobial, there have been, to the best of the authors’ knowledge, no studies showing that dressings impregnated with Manuka honey can kill organisms in the dressing itself. Materials and Methods. The investigators used the American Association of Textile Chemists and Colorists’ 100 test methodology to compare honey-impregnated dressings with control dressings (without honey) on the ability to kill common wound pathogens. Organisms were chosen after a review of the causal organisms found in actual wound infections over a 12-month period in a busy outpatient wound clinic. Results. Even when the dressings were challenged daily with further inoculated organisms, > 5-log reductions were routinely noted across a range of pathogens, including multiple drug-resistant species using dressings containing Manuka honey relative to the control. Conclusions. The results presented herein show that when well-characterized medical-grade Manuka honey is used in dressings (ie, a minimum of 400 mg methylglyoxal/kg) these dressings can comprehensively kill common wound pathogens associated with infected wounds.

Key words: Manuka honey, research, biology, topical agents

Wounds 2017;29(9):262–268. Epub 2017 June 28

While honey has been used for centuries to treat wounds, only relatively recent approvals have been given for use in “modern” wound care.1 Publications on the use of honey to treat a variety of wounds have increased, but a 2008 Cochrane review2 criticized the study design of many such publications. Conclusions from Jull et al2 about potential efficacy were moderately enthusiastic about honey with regard to partial-thickness burns, suggesting reduced healing time compared with conventional dressings; however, those were categorical in stating that honey used alongside compression therapy does not improve healing of venous leg ulcers. Molan3 and Brölmann et al4 were more enthusiastic, particularly about the specific use of Manuka honey, the latter showing that of 109 evidence-based conclusions (using Cochrane reviews), strong data supported the use of topical honey to reduce healing times.

An earlier review by Molan5 provided comprehensive evidence from clinical and animal studies that demonstrated the efficacy of honey in a wide variety of wound types and conditions. They noted that no large, double-blinded,
randomized trials had been carried out, and so, as per other antimicrobial dressing types, it was necessary to consider other trials and publications going back as far as the 1950s. Molan\(^5\) further explained that honey’s use in animals (eg, pigs, rats, buffalo calves, mice, and rabbits) was increasingly promising. These uses showed improvements in healing after surgical wounds compared with nonhoney treated wounds.

While undiluted honey is hypertonic to growing cells in vitro, no deleterious effects are seen in vivo, even when ingested by millions of people daily. Indeed, the high osmotic strength of honey contributes to its debridement action.

This contradictory evidence makes it difficult for modern practitioners to make rational decisions on the choice of treatment regimens; this is partly due to both the complexity of wound types assessed and the lack of standardization of honey used in modern medical dressings. A previous study\(^6\) evaluating the use of dressings (MANUKAhbd; ManukaMed USA, Franklin, TN) containing a potent medical-grade honey (>400 mg methylglyoxal [MGO]/kg) to treat 169 wounds of varying type in 150 patients over a 6-month period showed extremely favorable results, with only 27 wounds still in treatment after 6 months. To put this in perspective, these patients were referrals from other practitioners where the patients had nonresolving wounds prior to being treated with honey dressing.

Despite evidence that Manuka honey in vitro is strongly antimicrobial,\(^7\) to the best of the authors' knowledge there has not been a study showing that Manuka honey-impregnated dressings can kill organisms in the dressing itself. It is important that dressings left on wounds do not become “incubators” for further microbial growth resulting in re-infection of the wound. As such it is crucial that the organisms are killed in the dressing. The investigators report herein the use of a modified American Association of Textile Chemists and Colorists (AATCC; Research Triangle Park, NC) 100 test method (AATCC-TM100)\(^8\) comparing honey-impregnated dressings with control dressings (without honey) on the ability to kill a number of common wound pathogens, even when the dressings were challenged daily with further inoculated organisms. In this study, >5-log reductions were routinely noted across a range of pathogens, including multiple drug-resistant species.

**Materials and Methods**

All media and reagents used in the test were autoclave sterilized for at least 25 minutes at a minimum temperature of 121°C. All tests were carried out in triple replicate, and all enumerations were plated in double replicate. Microchem Laboratories (Round Rock, TX), an independent commercial laboratory, was the study laboratory.

**Test microorganisms.** The AATCC-TM100\(^8\) was used to assess sensitivity of the organisms to honey dressings. Organisms were chosen on the basis of an independent review of the causal organisms found in actual wound infections over a 12-month period in a busy outpatient clinic (Jackson-Madison County General Hospital, Jackson, TN).

The exception was *Candida* spp, an opportunistic pathogen.\(^9\) Test cultures were initiated either from a frozen freezer stock or a monthly working stock of isolated colonies. *Staphylococcus aureus* (BAA-42), *Klebsiella pneumoniae* (700603), *Pseudomonas aeruginosa* (BAA-2114), *Enterobacter cloacae* (BAA-2468), *Acinetobacter baumannii* (19606), and *Proteus mirabilis* (25933) (American Type Culture Collection, Manassas, VA) were each initiated independently in 10 mL tryptic soy broth and allowed to incubate at 36°C ± 1°C for 24 hours ± 4 hours. *Candida albicans* (T0231) (American Type Culture Collection) was initiated on solidified potato dextrose agar and allowed to incubate at 30°C ± 2°C for 48 hours ± 4 hours. Each culture was diluted independently in phosphate-buffered saline to achieve a test inoculum titer of 1 x 10⁶ colony forming units (CFU)/mL. A sterilized inoculating loop was used to scrape *C. albicans* colonies from the test culture and inoculate an aliquot of phosphate-buffered saline. Enough colonies were taken from the test culture plate and added to the aliquot to achieve a test inoculum titer of 1 x 10⁶ CFU/mL.

A second round of testing with *S. aureus* and *K. pneumoniae* included an added protein challenge in the test inocula. This protein challenge, a simulated wound fluid (SWF), was composed of 5.0% ± 0.2% bovine serum albumin, 142 ± 5 mM sodium chloride, and 2.5 ± 0.1 mM calcium chloride. The SWF was filter sterilized using a 0.2-µm filter to maintain the structure of the protein, which would be denatured in the case of steam sterilization. The test cultures were diluted in SWF instead of phosphate-buffered saline to achieve an inoculum titer of 1 x 10⁶ CFU/mL.

**Test substances and inoculation.** Honey dressings are commercially available sterile dressings approved by the US Food and Drug Administration (FDA) and Conformité Européenne and made of a proprietary super absorbent fiber. There are differences in the level of absorbent fibers and honey that the product line contains, with the lite version (MANUKAhbd Lite; ManukaMed, USA) being more suitable for wounds that exude less. The honey used is 100% New Zealand-sourced Manuka honey (*Leptospermum scoparium*) (Watson and Son, Masterton, New Zealand). The honey in the lite dressing had a MGO content of 496 mg/kg honey, while the regular honey dressing had
a MGO level of 449 mg/kg as determined by the method of Windsor et al.\textsuperscript{10}

The honey dressings were aseptically cut into 8-cm diameter circles so the carriers could fit in petri dishes and hold a large volume of liquid. The carriers were inoculated with 1 mL of the test inoculum prepared as described above. Each microorganism was tested independently. Parallel control carriers containing no honey were inoculated in the same fashion as the test carriers. Honey dressing test and control carriers were inoculated once daily for 7 consecutive days. For each of the 7 days, the carriers were incubated at 34°C ± 2°C for 24 hours ± 1 hour to mimic a real world scenario where a skin contact dressing takes on multiple challenges from an exuding wound over the course of 1 week. Carriers were harvested at different intervals over the course of the 7 days (after 1, 2, 3, and 7 challenges) to capture and enumerate the concentration of microorganisms at the time of harvest.

The lite honey dressings were aseptically cut into 50 mm x 50 mm carriers. These carriers were tested in the same fashion as the regular honey dressing carriers with the exception of fewer challenges. The lite honey dressing carriers experienced 3 challenge cycles once daily for 3 consecutive days instead of 7 and were harvested after 1, 2, and 3 challenges.

_**Harvesting of carriers and enumeration.**_ Honey dressing carriers were harvested in 30 mL of Deo-Engley broth in 50-mL conical vials. Lite honey dressing carriers were harvested in 20 mL of Deo-Engley broth in 50-mL conical vials. All carriers were vortex-mixed on maximum speed for 60 seconds ± 5 seconds. The resulting suspensions were then enumerated by serially diluting an aliquot from each suspension in phosphate-buffered saline to achieve a concentration of approximately 100 CFU/mL. These aliquots were then pour plated with the appropriate agar type to achieve growth of the test microorganisms. All plates poured with tryptic soy agar were incubated at 36°C ± 1°C for 48 hours ± 6 hours to capture bacterial growth. All plates poured with potato dextrose agar were incubated at 30°C ± 2°C for 48 hours ± 6 hours to capture fungal growth.

_Controls and neutralization verification._ Each lot of prepared medium or reagent was pour plated to assess sterility. All media and reagents were confirmed to be sterile for all conducted tests. Positive controls were also performed to ensure the test cultures were pure and the conditions and media were suitable for test microorganism growth. All positive controls yielded isolated colonies that demonstrated homogeneous colony morphologies.

The initial microorganism concentration on carriers was determined by inoculating control carriers in the same fashion as the test carriers and then immediately harvesting and enumerating as described above. This control confirmed the carriers were inoculated with at least 1 x 10\textsuperscript{6} CFU/mL at the beginning of the test and the microorganisms could adequately be recovered from the carriers using the harvesting technique previously described.

The barrier properties of the wound dressings were determined by a microbial ingress test. Each test and control substance type was placed on solidified growth agar as appropriate for the test microorganism. The carriers were inoculated with 0.1 mL of test inoculum to achieve a concentration of at least 1 x 10\textsuperscript{6} CFU/carrier. The carriers were allowed to incubate on the agar plates for at least 48 hours at an incubation temperature suitable for the microorganism involved. After the 48-hour incubation time was observed, the test and control carriers were removed from the agar to screen for microbial growth. All control carriers had microbial growth under the carriers on the agar. The test carriers demonstrated adequate barrier properties against bacterial growth and prohibited the inoculum from penetrating the dressing and allowing bacteria to grow on the agar. Both test substances did not show adequate barrier properties against _C. albicans_; however, the inoculum was not totally eradicated in the dressing because there was a small amount of growth present on the agar.

Neutralization of the test substance is required so the reaction between the active antimicrobial ingredient and the microorganisms can be halted when the carriers are harvested. Neutralization was confirmed by placing a carrier of each test substance in Dey-Engley broth of the appropriate volume for that type of test substance. The suspensions were then inoculated with enough microorganisms to achieve a concentration of approximately 100 CFU/mL. The suspensions were vortex-mixed and allowed to stand undisturbed for a minimum of 10 minutes. Two 1-mL aliquots were then removed and separately pour plated to assess the concentration of surviving microorganisms. Control suspensions were inoculated and enumerated similarly, though they contained control carriers with no Manuka honey. The enumeration counts of the test and control neutralization suspensions were compared and used to verify that active antimicrobial ingredient neutralization was sufficient. Neutralization test counts must be ≥ 70% of neutralization control counts for the test substance to be considered verified. The neutralization method was verified to be sufficient for every microorganism listed in the “Test Substances and Inoculation” section.
Calculations:

\[ \text{CFU/mL} = \text{average CFU/plate} \times \text{dilution factor} \]

\[ \text{CFU/carrier} = \text{average CFU/plate} \times \text{dilution factor} \]

Percent reduction is calculated as follows:

\[ P_1 = 100 \left[ \frac{B - A}{B} \right] \]

\[ P_1 = \text{percent reduction} \]

\[ A = \text{mean number of surviving microorganisms on test carriers} \]

\[ B = \text{mean number of surviving microorganisms on inoculated control carriers at time 0 (initial numbers control)} \]

Log reduction is calculated as follows:

\[ L_1 = \log \left( \frac{B}{A} \right) \]

\[ L_1 = \text{log reduction} \]

\[ A = \text{mean number of surviving microorganisms on test carriers} \]

\[ B = \text{mean number of surviving microorganisms on inoculated control carriers at time 0 (initial numbers control)} \]

Results

Table 1 is a summary of the \(\log_{10}\) reduction after 24 hours for various wound pathogens versus initial control numbers for both of the honey dressings. Comprehensive killing (> 5-log\(_{10}\) reduction) is apparent for all organisms when seeded to honey dressings; very similar results are shown for lite honey dressings, with an exception of \(C \ albicans\) where just under 4-log\(_{10}\) reduction is recorded. Although \(C \ albicans\) is not a common wound organism, it is included as it is associated with infections in burns patients.

\(S \ aureus\) and \(K \ pneumoniae\) were tested using SWF, a prerequisite for certain applications to the FDA. As the name suggests, SWF attempts to simulate conditions existing in vivo by incorporating protein in the reaction milieu. In the presence of SWF, all log\(_{10}\) reductions, and therefore killings, were lower in both organisms (compared with when undertaken without SWF), but this was marginal for all combinations except \(S \ aureus\) and the honey dressing where the presence of SWF reduced the killing by 1.49 log\(_{10}\) units. Nevertheless, a > 4-log\(_{10}\) reduction for honey dressings in the presence of SWF represents a potent killing.

Tables 2 and 3 show the full detail of killing for \(P \ aeruginosa\) (BAA-2114).

Discussion

The investigators carried out this trial with plans to submit the results in support of a 510K FDA antimicrobial dressing claim. In such a submission, the FDA only considers in
situ killing in the dressing to be relevant to the application. This, of course, is only part of the clinical story. Observations by wound care nurses and practicing physicians will attest that the dressings absorb exudate (and gain weight as proof) and the honey moves from the dressing into the wound bed. This outcome forms the basis of the dressing’s honey delivery system. In this paper, the investigators are not addressing the killing of microorganisms within the wound, but clinical evidence is uniformly in agreement that reduction/elimination in the cause of the infection is necessary to begin the healing process; thus, killing organisms both in the dressing and the wound bed are crucial.11

Tables 1 and 2 present overwhelming evidence that in situ killing of these pathogens is real and reproducible for both types of honey dressings. To the best of the investigators’ knowledge, this is the first time such a study has been undertaken using medical-grade honey and a modified AATCC-TM100 with a range of relevant pathogens. Greater than 5-log reductions within 24 hours for 6 important and common wound pathogens were noted. With the lite honey dressing, the only organism that showed less sensitivity in this assay was *C. albicans*. This combination showed just under a 4-log reduction. The investigators did not explore the reasons for this reduced sensitivity to the lite honey dressing.

### Table 2. Detailed data on the efficacy of the honey dressing against *Pseudomonas aeruginosa* (BAA-2114)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Challenge Time</th>
<th>Test Surface</th>
<th>Replicate</th>
<th>Replicate CFU/carrier</th>
<th>Avg CFU/carrier</th>
<th>Percent Reduction vs. Initial No. Control</th>
<th>Log Reduction vs. Initial No. Control</th>
<th>Percent Reduction vs. Relative Control</th>
<th>Log Reduction vs. Relative Control</th>
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<tr>
<td><em>P. aeruginosa</em> BAA-2114</td>
<td>Initial No. Control</td>
<td>Oasis 2649</td>
<td>1</td>
<td>8.55E+06</td>
<td>1.04E+07</td>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<td>24h</td>
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<td>&gt;5.84</td>
<td>&gt;99.9995%</td>
<td>&gt;5.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Honey dressing</td>
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<td>&lt;15</td>
<td>&lt;15</td>
<td>99.9999%</td>
<td>&gt;5.84</td>
<td>&gt;99.9995%</td>
<td>&gt;5.33</td>
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<td>&gt;5.84</td>
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<td>&lt;15</td>
<td>99.9999%</td>
<td>&gt;5.84</td>
<td>&gt;99.99992%</td>
<td>&gt;7.09</td>
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<td></td>
<td>72h</td>
<td>Oasis 2649</td>
<td>1</td>
<td>2.00E+08</td>
<td>2.02E+08</td>
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<td>&gt;99.999993%</td>
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<td>N/A</td>
<td>N/A</td>
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<td>&lt;15</td>
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<td>&lt;15</td>
<td>&lt;15</td>
<td>99.9999%</td>
<td>&gt;5.84</td>
<td>&gt;99.999998%</td>
<td>&gt;7.67</td>
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</tbody>
</table>

Note: The limit of detection for this study is 15 CFU/carrier; results < 15 CFU/carrier are reported as such. CFU: colony forming unit.
**Table 3.** Detailed data on efficacy of the lite honey dressing against *Pseudomonas aeruginosa* (BAA-2114)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Challenge Time</th>
<th>Test Surface</th>
<th>Replicate</th>
<th>Replicate CFU/carryer</th>
<th>Avg CFU/carryer</th>
<th>Percent Reduction vs. Initial No. Control</th>
<th>Log Reduction vs. Initial No. Control</th>
<th>Percent Reduction vs. Relative Control</th>
<th>Log Reduction vs. Relative Control</th>
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<tr>
<td><em>P. aeruginosa</em> BAA-2114</td>
<td>Initial No. Control</td>
<td>Oasis 2253</td>
<td>1</td>
<td>9.70E+06</td>
<td>9.53E+06</td>
<td>N/A</td>
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<tr>
<td></td>
<td>24h</td>
<td>Oasis 2253</td>
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<td>1.33E+08</td>
<td>1.30E+08</td>
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<td>&gt;99.9999%</td>
<td>&gt;5.98</td>
<td>&gt;99.9999%</td>
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<td></td>
<td>Lite honey dressing</td>
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<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&gt;99.9999%</td>
<td>&gt;5.98</td>
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<td>&gt;7.11</td>
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<td>48h</td>
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<td>2.06E+08</td>
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<td>&gt;99.9999%</td>
<td>&gt;5.98</td>
<td>&gt;99.9999%</td>
<td>&gt;7.39</td>
</tr>
</tbody>
</table>

Note: The limit of detection for this study is 10 CFU/carrier; results < 10 CFU/carrier are reported as such. CFU: colony forming unit

*C. albicans* is a dimorphic organism existing in a yeast-like, filamentous form depending on environmental conditions. The investigators noted the yeast form under microscopic examination, but it is possible that both forms exist to some extent when inoculated onto the dressing and that one form is more sensitive to Manuka honey than the other. Mitchell also found both forms may have a role in infection, but equally Lo et al suggest only the filamentous forms are virulent. Either way, the dimorphic nature of *C. albicans* may account for why there is some growth in the microbial ingress tests with this organism.

While there is a plethora of in vitro methods for assessing the ability of a substance to kill microorganisms, all tests depend on contact time, concentration, temperature, pH, the number and type of test organism, and the presence of interfering substances. In addition, the nature of the media in which the organisms are grown may induce phenotypic variation. The investigators standardized for the above and showed that even in the presence of SWF to simulate the wound environment, *S. aureus* (BAA-42) and *K. pneumoniae* (700603) showed largely similar sensitivity to the honey dressings as those assays when SWF was not used (Table 1).

In reviewing other methodologies, Gallant-Behm et al were highly critical of disc diffusion assays as a measure of antimicrobial wound dressing efficacy, arguing that such assays have no means to quantify the level of the antimicrobial agent released from the dressing. They advocated for the use of the logarithm reduction method, akin to what was used in the study herein. The present results made no attempt to measure release of honey from the dressing, but the investigators concur that this logarithm reduction methodology is appropriate for assessing in situ killing within a dressing.

The 6 pathogens used, including multiple drug-resistant strains, showed remarkably similar and high sensitivity to medical-grade honey dressings. Dilution of the honey...
within the dressing will of course take place in vivo as the exudate is absorbed into the dressing due to osmotic pressure. However, there seems to be sufficient evidence that since medical-grade honey is approximately 80% (w/w) on a solid basis, antimicrobial killing is retained for most pathogens even at or below 10% (w/w)\textsuperscript{16,17}; for at least one \textit{Pseudomonas} strain, this may be as high as 30\%.\textsuperscript{18} Either way, there seems to be significant scope for honey dilution within the dressing without loss of killing efficacy.

**Limitations**

The major limitation of this work is that it only measured killing within the dressing itself, not in the wound bed. A future study could address this, because both are essential for wound healing. Further, while microbial killing is a \textit{sine qua non} to effective healing, medical-grade Manuka honey clearly contains factors that promote granulation; this is a fruitful avenue for future research.

**Conclusions**

The investigators believe the results presented herein, despite in vitro data only, lend credence to the notion that when well-characterized medical-grade honey is used (a minimum of 400 mg MGO/kg is recommended), honey dressings can comprehensively kill common wound pathogens associated with infected wounds. Given Manuka honey’s evident lack of toxicity (global edible consumption is > 2000 tons annually, plus a study showing lack of toxicity\textsuperscript{19}), an inability to induce any sort of resistance to Manuka honey in bacteria tested to date,\textsuperscript{20} and affordability, it is time to encourage a greater use to reduce the morbidity\textsuperscript{21} and the cost associated with wound care infections in the United States.

**References**