Extraction of DNA from Honeybee, *Apis mellifera*, Beeswax for Genetic Analysis of the Microbiome

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Abstract

Beeswax has the potential to harbor a diverse microbiome that may be an indicator of honeybee colony health. In order to characterize the microbiome with a culture-independent method, DNA must first be extracted from the beeswax, amplified by PCR, and sequenced. Currently, there is no established protocol for DNA extraction from beeswax. The properties of beeswax can make DNA extraction difficult and inhibit PCR. To find an effective protocol of DNA extraction for sequencing, various beeswax sampling tools were used in combination with different DNA extraction methods in this study. Samples were then assessed for their ability to amplify by PCR. Multiple methods yielded amplification by PCR with varying degrees of success.

Introduction

The honeybee, *Apis mellifera,* is an important financial asset for beekeepers in addition to being essential for the agricultural industry via pollination (Calderone, 2012). However, the Bee Informed Partnership (BIP) National Colony Loss and Management Survey reported that US beekeepers experienced an estimated loss of 32.2% of their colonies during the winter of 2020-2021 (Steinhauer et al., 2021). The current consensus for the cause of colony loss is a conglomerate of stress factors such as parasitic mites, insecticides, food shortages, weather, and pathogens (Neumann & Carreck, 2010).

While studies have been conducted on pathogens in the hive environment, these studies are often of small scope, only utilize culturing methods, and focus on one pathogen rather than the entire microbiome. Technologies exist to sequence DNA and identify microorganisms found within the microbiome; however, these methods have not been readily used for investigating the microbiome of beeswax. This is likely due to difficulties in extracting DNA from wax.

DNA extraction methods use the chemical properties of DNA and contaminants to yield purified DNA for downstream use in PCR or sequencing. Various methods and commercial kits are available for DNA extraction. Kits, such as Qiagen's DNeasy PowerSoil Pro Kit and DNeasy PowerFood Microbial Kit, are designed to extract DNA from certain sample types and to remove specific contaminants from those samples that would inhibit PCR. Numerous substances, including proteins, polysaccharides, phenols, and lipids, can inhibit PCR and prevent amplification of the 16S region used for DNA sequencing (Richards, 1999). While the composition can vary, beeswax contains hundreds of compounds, including a large amount of lipids and proteins (Hepburn, 1986). Due to the hydrophobic nature of lipids, problems can occur in DNA extractions that rely on aqueous solutions. The properties of lipids complicate not only the separation of microorganism cells from the beeswax but also the removal of PCR inhibiting contaminants from the sample. Preliminary attempts at DNA extraction from beeswax have resulted in excess contamination or the degradation and loss of DNA from multiple purification steps.

The goal of this study is to assess multiple sampling tool types and DNA extraction methods to optimize a protocol for DNA extraction from beeswax. This protocol can then be used to analyze the hive microbiome and gain better insight into honeybee health and unexplained colony loss.

Methods

Sampling

To determine the best area for microbiome sampling within the interior hive environment, samples were taken with a sterile cotton swab from the hive entrance, the top of a frame, the interior wall of a brood box, the backs of bees, beeswax covering the brood, and inside an open cell. The swabs were sonicated in 0.01% $MgSO_4$ buffer and spiral plated on nutrient-rich agar. The plates were incubated for 3 days at 37° C, and bacterial colonies were counted. Samples taken from beeswax covering brood produced the highest number of colonies (Fig. 1), likely due to the high amount of contact with bees and high nutrient content from the beeswax composition. Beeswax covering brood was, therefore, determined to be the best area to focus on for this study.

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Samples were taken from the beeswax covering brood of five unrelated honeybee colonies. Three types of sampling tools, a dry sterile cotton swab, a cotton swab saturated with 0.01% $MgSO_4$ buffer, and autoclaved flat wooden picks, were selected for use. Both dry and buffered cotton swabs were twirled with moderate pressure over the beeswax covering brood in an 8x8 cm area for 30 seconds. The wooden picks were inserted into the beeswax covering brood to remove the capping containing approximately 100mg to 250mg of beeswax. Dry swabs and the wooden pick samples were stored in microcentrifuge tubes and temporarily kept on ice until placed in a -80° C freezer. Buffered swabs were kept on ice and underwent DNA extraction within two hours after sampling.

DNA Extraction

Four different DNA extraction methods were used in this study. The Qiagen DNeasy PowerSoil Pro Kit was selected since it is designed for small quantities of DNA, uses a bead beating tube for spore extraction and a spin filter column, and removes most PCR inhibitors. The Qiagen DNeasy PowerFood Microbial Kit was selected as it is designed for small DNA quantities and uses a bead beating tube and a spin filter column. It is designed to remove PCR inhibitors specific to foods such as lipids. The Bio-Rad InstaGene Matrix, which utilizes chelating particles to remove PCR inhibitors, was selected for both cost and ease of use. Crude preparation was also selected for cost and ease of use. The Qiagen PowerSoil Pro Kit, Qiagen PowerFood Microbial Kit, and Bio-Rad InstaGene Matrix were used according to manufacturer directions for each sample tool type for all 5 honeybee hives sampled. An exception is that 400ul instead of 200ul of InstaGene Matrix solution was used per sample to ensure coverage of the swabs and to increase inhibitor removal capacity. Furthermore, the wooden pick or cotton swab was used in place of soil or food for the respective kit. The crude preparation of DNA extractions involved adding 500ul of nuclease free water to the samples, vortexing, and boiling at 101º C for 10 minutes. Samples were vortexed again and centrifuged at 12,000g for 2 minutes. The supernatant was used as a PCR template. All samples, except crude preparation samples, were analyzed by NanoDrop for DNA concentration and purity.

PCR

The 16S rRNA gene PCR amplification was performed using universal primers 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplifications were carried out under the following conditions: initial denaturation at 95°C for 10min, followed by 35 cycles of denaturation at 95°C for 15 seconds, primer annealing at 50°C for 60 seconds, and extension at 72°C for 120 seconds, with a final elongation at 72°C for 10 min. For each PCR reaction, the following was used: 5ul of template, 1.5ul $MgCl_2$, 5.0ul buffer, 0.5ul dNTPs, 2.0ul 27F primer, 2.0ul 1492R primer, 0.20ul Taq polymerase, and 8.5ul nuclease free water.

Results

Culturing of the samples from different areas within the hive yielded varying results (Fig. 1). Samples from beeswax covering brood produced the largest number of bacterial colonies with at least three distinct morphologies seen. There were no bacterial colonies obtained from the wooded surfaces in the interior of the hive, although divots were observed in the media. Diverse bacterial colonies were obtained from the hive entrance; however, more growth was seen for these samples at 27°C than 37°C, indicating, along with the morphology of some of the colonies, that these bacteria were likely environmental species and not honeybee symbionts or pathogens.

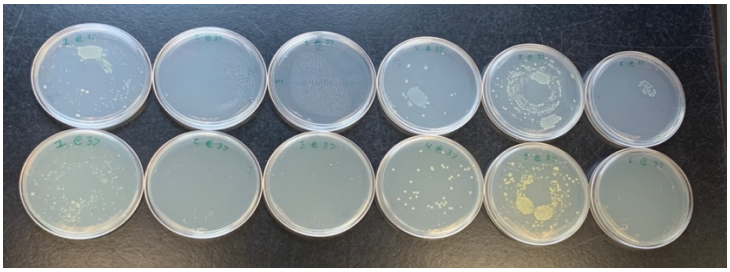

Fig. 1. Cultures from various areas of the honeybee hive. The top row is minimal media, and the bottom row is nutrient-rich agar. From left to right, the samples were taken from the hive entrance, top of the frame, wall of the brood box, backs of bees, beeswax covering capped brood, and inside an open cell.

Gel electrophoresis was used to confirm that after extraction, samples could be amplified by 16S PCR (Fig. 2). All samples taken with any of the three sampling tools used with the Qiagen PowerSoil Pro Kit were able to be amplified. Samples used with Qiagen PowerFood Microbial Kit were able to be amplified when samples were taken with a wooden pick or a dry cotton swab. The buffer saturated swabs had mixed success, with only 3 out of 5 samples amplifying. DNA extraction with InstaGene Matrix produced minimal amplification with the dry cotton swab and no amplification with wood or buffered swabs. Crude preparation did not produce any amplification. The band seen for dry swabs under C2 in Fig. 2 was due to a gel loading error.

Bands showing that the 16S rRNA region of bacteria was able to be amplified appeared for the negative controls for all sample collection tool types. The negative controls, in this case, consisted of an unused swab or wooden pick. Sanger sequencing of the negative controls showed that the contamination came from shared lab equipment.

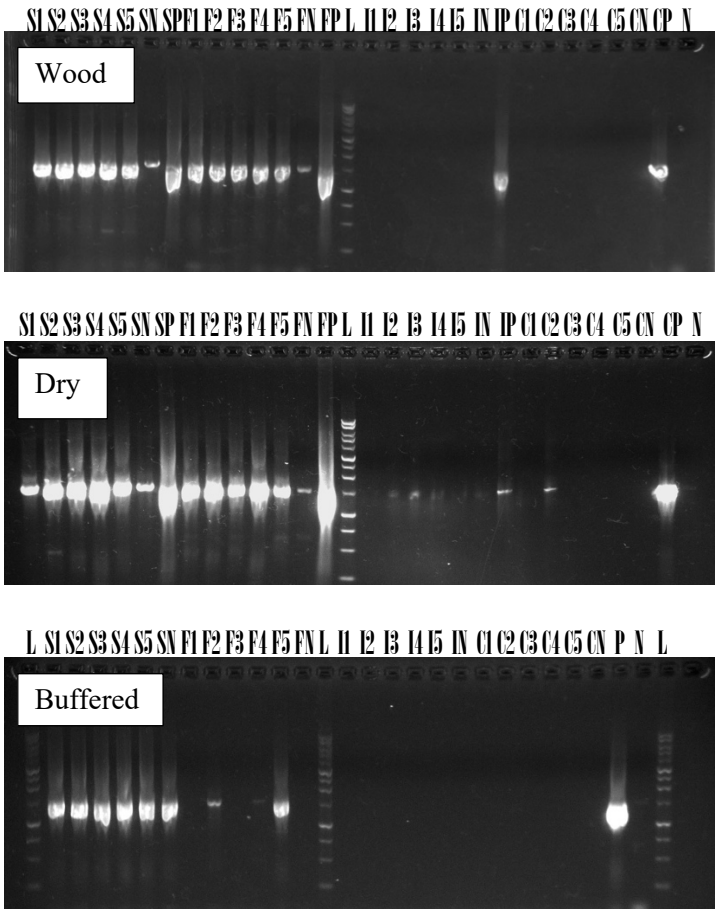

Fig. 2. Gel electrophoresis results of 16S PCR, where S refers to samples extracted with Qiagen PowerSoil Pro Kit, F refers to Qiagen PowerFood Kit, I refers to Bio-Rad InstaGene Matrix, C refers to crude preparation, P refers to the positive control, N refers to a negative control, and L refers to a 1kb ladder. A white band at around 1,500bp indicates successful amplification of the 16S rRNA gene region. Note that the brightness of bands does not indicate amplified DNA quantity. The gel for dry swabs was overexposed to better visualize the InstaGene bands.

Before PCR, samples were analyzed for DNA concentration and purity with NanoDrop. Table 1 lists the average concentration of DNA in ng/ul for sample tool type and DNA extraction method. On average, the samples collected with wooden picks contained more DNA than the other sampling tools. The dry swabs and buffered swabs had comparable amounts of DNA. It should be noted that the $MgSO_4$ buffer contained bacterial contamination that may have inflated the amount of bacteria, and therefore DNA, that can be collected by a buffer-saturated swab. Samples processed with the Qiagen PowerFood Microbial Kit had a DNA concentration double that of the Qiagen PowerSoil Pro Kit. Although the InstaGene method produced a high concentration of DNA, this method did not yield consistent PCR results.

Table 1. Average DNA in ng/ul extracted from the Qiagen PowerSoil Pro Kit, the Qiagen PowerFood Kit, and InstaGene Matrix for the various collection tool types.

	Soil Kit	Food Kit	InstaGene
Dry swab	3.62	6.92	115.26
Buffered swab	3.28	7.00	100.64
Wood	5.80	18.96	608.94

The presence of contamination was determined by NanoDrop spectroscopy through 260/280 and 260/230 absorbance ratios. The average readings for samples that could be amplified by PCR were 17.1 ng/ul with a 260/280 ratio of 1.88 and a 260/230 ratio of 0.15. The average readings for samples that were not amplified were 218 ng/ul with a 260/280 ratio of 1.15 and a 260/230 ratio of 0.41. A T-test determined the readings for concentration, 260/280 ratio, and 260/230 ratio to be significantly different between the samples that were able to be amplified by PCR and the samples that were not able to be amplified ($p < 0.005$).

A sample taken with a dry cotton swab extracted with the Qiagen PowerSoil kit was able to be sequenced using Illumina Miseq with an acceptable (>10,000) number of reads. Samples used with other extraction methods have not yet been submitted for sequencing.

Discussion

The resulting bacterial colony growth from the different hive areas leads to further questions that can be investigated in future studies. Investigating why there are no culturable bacterial colonies from the interior wooden surfaces of the hive could lead to new knowledge regarding the i-microbial properties of the hive environment. Further studies concerning the bacterial composition of the microbiome found at the hive entrance can provide insight into the vector ecology of honeybees as carries of environmental bacteria such as those that may be plant pathogenic.

Although there appears to be bacterial contamination in all DNA extraction samples, this serves to demonstrate which DNA extraction methods can be successful when bacteria are known to be present. Among the protocols tested, using a wooden pick or dry cotton swab and either the Qiagen PowerSoil Pro Kit or Qiagen PowerFood Microbial Kit was the best option for ensuring amplification of the 16S rRNA gene region, which can then be used for sequencing. Since bacteria are not highly abundant inside the hive, using a wooden pick that can collect a larger sample and the Qiagen PowerFood Microbial Kit, which yielded the highest DNA concentrations of the methods that worked, maybe the best option for assessing the microbiome. If cost is a factor, the Qiagen PowerFood Microbial Kit is the more economical option compared to the Qiagen PowerSoil Pro Kit. It should

be noted that the bead beating tubes in the Qiagen PowerFood Microbial Kit is too small for the swabs or wooden picks. The beads may be transferred to the microcentrifuge tube used for sample collection. The inconsistent results of the Qiagen PowerFood Microbial Kit, when used with the buffered swabs, show that there may be an incompatibility with the kit and $MgSO_4$ and, therefore, should not be used together.

Wooden picks may sample more invariably than cotton swabs. Unless the wooden picks are weighed before, and after sampling to record the mass of beeswax taken, the samples may vary significantly. Meanwhile, swabbing an 8x8 cm area for 30 seconds generally saturates a dry swab with wax and normalizes the samples.

Results indicate that higher concentrations of DNA after extraction do not indicate a greater likelihood of being amplified by PCR. Instead, lower concentrations of extracted DNA are associated with amplification ability and may indicate a higher degree of purification. However, 260/280 ratios seem to be most indicative of the ability to amplify. A reading of 1.8 for a 260/280 ratio is considered pure for DNA (Thermo Scientific, 2012). The samples that were able to be amplified had an average 260/280 ratio of 1.88, while the samples that were unable to amplify had a ratio that was significantly lower. A ratio of 2.0 or more for 260/230 ratios indicates pure DNA, with lower values indicating contamination. The 260/230 ratios seen in this study were all significantly lower than 2.0. However, this did not affect the ability to amplify; in fact, the group that was able to be amplified had a lower 260/230 ratio. Low 260/230 ratios were found to have no significant effect on the ability of RNA samples to successfully undergo qRT-PCR (Cincinnati et al., 2008). In this case, low 260/230 ratios can be disregarded as an indicator of potential PCR inhibition.

While previously, there has not been an established protocol for DNA extraction from honeybee beeswax, this study shows that DNA extraction from beeswax, 16S rRNA gene amplification, and sequencing of the hive microbiome is possible. This will allow for future studies of the hive microbiome that may show the microbiome to be an indicator of honeybee colony health.

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Kylie S. Weis is a third-year student pursuing a B.S. degree in Applied Biotechnology at Auburn University. She has led key research roles in experimental design, sampling, lab work, and analysis for this project. She has a background in plant pathology and plans to continue her education by researching the genetic components of plant-microbe interactions.

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Geoffrey Williams, Ph.D. is a Professor at Auburn University, where he began working in 2016. He earned his Ph.D. in Biology at Dalhousie University, Canada. He is the President of the Bee Informed Partnership and on the executive board of COLOSS. His work includes studying the effects of parasites and pesticides on honeybee health.