

# Characterization of the Normal Flora of Honeybee Hives in Central Wisconsin

Jordan L. Bendel

Faculty Sponsor: Bonnie Jo Bratina, Department of Microbiology

## ABSTRACT

Honey bees have played an important role in nature for years. Presently, honeybees play a major part in agriculture and business in the United States. In recent years, the beekeeping industry has been faced with several problems ranging from antibiotic resistant pathogenic bacteria to parasitic varroa mites. Although a great quantity of research has been done on bacteria that are pathogenic to honey bees, very little is known about the normal flora of the bee hive and its role in the hive. We are characterizing the predominant species of bacteria in beehives in Central Wisconsin. We have collected 31 isolates from bee hives, honeybees and also from varroa mites. Of these 31 isolates, seven were gram negative, catalase positive, and urease negative (three of which were also oxidase positive). Six of these gram negative organisms were rods with only one cocci. We also found 24 gram positive, catalase positive bacteria (five of which were oxidase positive). Eighteen of the 24 were rods and six were cocci. Currently the 16S rRNA gene from the isolates is being sequenced to determine their phylogenetic affiliation. Trials with these bacteria on honey bee colonies to see if any of the isolates were pathogenic to varroa mites were inconclusive. Characterizing the normal flora and how they interact could possibly be a first step toward solutions to some of the industry's problems.

## INTRODUCTION

Varroa mites first arrived in the United States in 1987, and were discovered in a beehive in Wisconsin (5). Within two years they had spread throughout the U.S. and were devastating hives by killing trillions of bees, the varroa mite was able to put some beekeepers out of business. By 1990 the beekeeping industry was up in arms about the whole situation and most thought it was the end of the domestic honeybee in the U.S.

In 1991 fluvalinate, a miticide, was put in the market to combat the varroa mites. Most thought it was the savior for the industry, but due to improper use by 1998 complete colonies of mites were showing resistance to fluvalinate (5). This led to major losses that winter, ranging from 90-100% of hives in apiaries throughout the United States. Losses in bees, pollination of crops, and honey production due to the infestation of mites now started to climb into the millions of dollars (2). This led to the development of a different miticide, coumaphos (6). However, due to its toxicity to humans, many beekeepers would like to avoid its use. Nevertheless some beekeepers depend on their bees to support them, and so have to depend on coumaphos.

There has been a lot of work done on the bacterial control of other mite species that destroy a variety of agricultural goods. This research has shown that bacteria such as *Wolbachia* or *Bacillus thuringiensis* are able to control these mite species (3,4). In my research I intend to try to isolate a bacterium that is pathogenic to the varroa mite.

## METHODS

**Mite collection.** Mites were collected in Vernon County, Wisconsin from seven different apiaries containing 1-20 hives each. One to two hives were sampled at each site for the presence of varroa mites. Mite collection was performed using a mason jar with a 1/8 inch screen secured to the opening of the jar. Honey bees were funneled into the jar until about 100-300 honey bees were collected. The screen was then put on and approximately 4-5 tablespoons of talcum powder was added to the jar. The bees were then evenly coated with talcum powder and allowed to sit for a couple of minutes. The jar was inverted over a whirl-pak and shaken causing the mites to be dislodged for collection. The talcum powder was then screened to retrieve the mites from the mixture. The mites were then rinsed with phosphate buffer to remove surface bacteria.

**Isolation of bacteria.** The rinse solution was serially diluted to  $10^{-3}$ . Each dilution was plated on Tryptic soy agar (TSA) and blood agar plate (BAP) to allow as many types of bacteria to grow as possible. Likewise, the washed mites themselves were then ground up and diluted to  $10^{-4}$  and plated. The plates were incubated at  $35^{\circ}\text{C}$  for 48 hours. The plates were checked for growth, and 2-8 different colonies were selected from each mite suspension. Swabs of the internal surfaces of the hives were also taken and plated on TSA and BAP. A total of 2-3 bacterial colonies were selected from each hive. Bacteria were chosen depending on differences in colony morphology or type of hemolysis seen on the BAP. The isolated bacteria were then streaked for isolation.

**Bacterial isolate characterization.** All bacterial isolates were checked for Gram reaction, morphology, oxidase and catalase reactions, anaerobic growth, and motility (1). Motility was determined using both wet mounts and Gard plates. In addition all gram positive bacteria were tested for glucose utilization, nitrate reduction, and Methyl Red-Voges-Proskauer reaction. The gram negative bacteria were tested for nitrate reduction, and citrate utilization (1).

**Mite pathogenicity trial.** An assay was formulated to check bacterial isolates for patho-

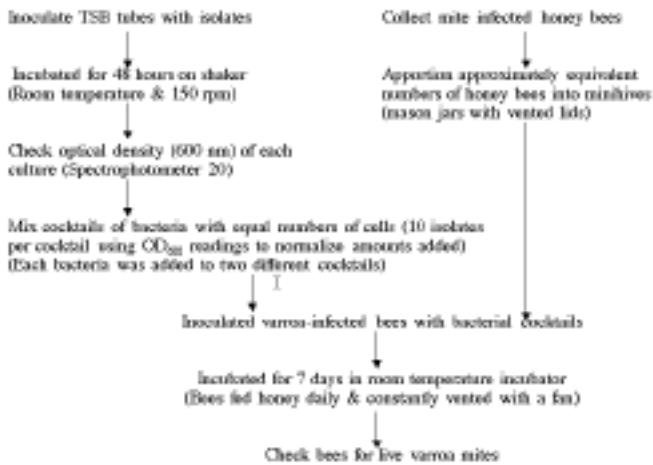


Figure 1. Flow chart of the assay used to test for pathogenicity of the bacterial isolates against varroa mites on honey bees.

genicity towards varroa mites (Fig. 1). Modifications to this assay included moving the bees from the minihives into larger boxes. This allowed the bees to have a bigger space and so could regulate the moisture and were less stressed. The larger hives were also placed in a 4 seasons porch to allow for better temperature control of the hives.

**Table 1. Characteristics of selected bacteria isolated from hives and mites from Vernon County, WI.**

Isolate	Sample site <sup>a</sup>	Colony description <sup>b</sup>	Gram S Stain	Form	Oxidase
V5	Rokosy	yellow/entire/flat/circular	negative	cocci	negative
V10	Rokosy	brown/entire/raised/circular	negative	rod	negative
V29	Bendel	brown/undulate/raised/irregular	negative	rod	positive
V44	Rokosy	tan/entire/raised/circular	negative	rod	positive
V47	Dale	cream/entire/flat/circular	negative	rod	positive
V52	Schultz	white/filamentous/flat/irregular	negative	rod	negative
V8	Rokosy	cream/undulate/convex/circular	negative	rod	negative
V20	Bendel	white/entire/flat/circular	positive	cocci	negative
V25	Rokosy	white/undulate/umbonate/circular	positive	cocci	negative
V3	Rokosy	cream/entire/umbonate/circular	positive	cocci	negative
V31	Bendel	yellow/undulate/flat/irregular	positive	cocci	negative
V35	Rokosy	cream/entire/umbonate/circular	positive	cocci	negative
V50.5	Rokosy	pink/entire/convex/circular	positive	cocci	negative
V9	Rokosy	tan/entire/umbonate/circular	positive	cocci	negative
V1	Rokosy	white/undulate/umbonate/circular	positive	rod	positive
V11	Rokosy	white/entire/flat/circular	positive	rod	negative
V18	Bendel	white/entire/convex/circular	positive	rod	negative
V2	Rokosy	yellow/entire/flat/circular	positive	rod	negative
V27	Rokosy	white/entire/flat/circular	positive	rod	negative
V28	Bendel	yellow/entire/flat/circular	positive	rod	negative
V30	Bendel	yellow/entire/convex/circular	positive	rod	negative
V32	Bendel	tan/entire/umbonate/circular	positive	rod	negative
V34	Rokosy	tan/entire/flat/circular	positive	rod	negative
V36	Rokosy	red/entire/raised/circular	positive	rod	negative
V37	Rokosy	orange/entire/convex/circular	positive	rod	negative
V4	Rokosy	white/entire/convex/circular	positive	rod	positive
V45	Rokosy	cream/entire/raised/circular	positive	rod	negative
V49	Gilbertson	white/lobate/flat/irregular	positive	rod	positive
V50	Langcamp	white/lobate/flat/rhizoid	positive	rod	negative
V6	Rokosy	white/undulate/umbonate/circular	positive	rod	positive
V7	Rokosy	light yellow/entire/flat/circular	positive	rod	negative

<sup>a</sup>Bendel, Rokosy, Gilbertson, Langcamp, and Dale refer to the location where the hives are kept.

<sup>b</sup>Colony descriptions were from TSA plates after a 24 hour incubation at 35oC.

**RESULTS**

Initially 55 isolates were recovered from TSA and BAP. Initial characterization of the isolates divided them into 6 groups. Characterization began by determining Gram stain, colony description, morphology, oxidase and catalase reaction (Table 1). The gram positive bacteria were checked for glucose utilization, nitrate reduction, and Methyl Red-Voges-Proskauer reaction. The gram negative bacteria were tested for nitrate reduction, and citrate utilization. This allowed the list of possible genera for each isolated to be narrowed down to 1-3 possible genera (Fig. 2 & 3). An assay was devised to allow for the determination of pathogenicity (Fig. 1). The first two trials were inconclusive for pathogenicity to mites. This was due to humidity problem with enclosed incubators which caused great stress to the bees. This problem was fixed by moving the bees into larger hives in an outdoor enclosed environment. The third trial was inconclusive due to cold weather killing four of the test hives. This problem was solved by moving the hives into a four seasons porch where the temperature of the hives could be better regulated. The fourth trial was also inconclusive due to low mite concentrations which made the results statistically inconclusive. After each trial any bacteria that were found to be pathogenic to honeybees were discarded from the study. After the last set of pathogenicity trials, 31 bacterial isolates were left to be characterized. Currently the 16S rRNA of remaining 31 isolates are being partially sequenced to determine either their exact genus and species if possible or at the least their phylogenetic affiliation

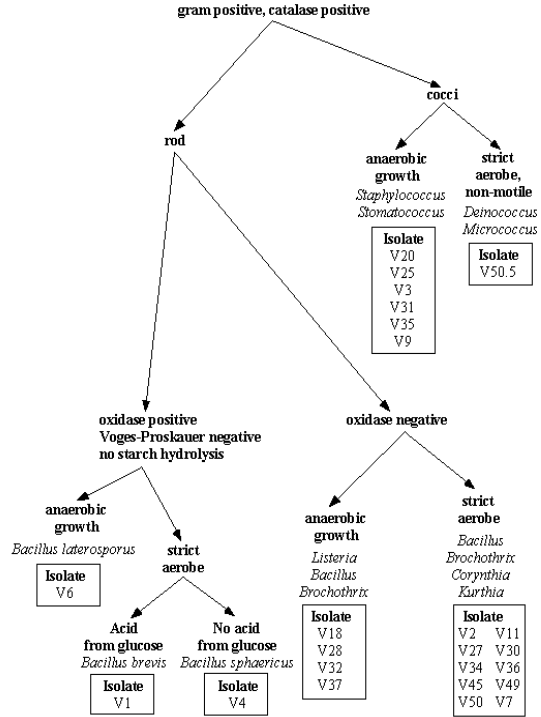


Figure 2. Test results used to determine possible genera of unknown gram positive isolates

**SUMMARY**

Thirty-one isolates from 13 hives in Vernon County, WI were partially characterized. These isolates showed no pathogenicity to honeybees. Of the 31 isolates, 24 were gram positive and 7 were gram negative. Of these 24 gram positive isolates, 14 were oxidase negative rods indicating these isolates may belong to the genera *Bacillus*, *Listeria*, *Brochothrix*, *Corynthia*, or *Kurthia*. Three of the isolates were gram positive, oxidase positive rods. The

genus for these isolates is most likely *Bacillus*. The last seven gram positive isolates were nonmotile cocci, and so possible genera for these isolates include *Staphylococcus*, *Stomatococcus*, *Micrococcus*, or *Deinococcus*.

Of the seven gram negative bacteria, three were oxidase positive rods, indicating these isolates may belong to the genera *Vibrio*, *Aeromonas*, or *Pseudomonas*. One of the gram negative bacteria was found to be an oxidase negative cocci whose genus is likely to be *Acinetobacter*.

The last three gram negative bacteria were oxidase negative rods which likely belong to the genera *Citrobacter*, *Salmonella*, *Gluconobacter*, or *Acetobacter*.

The pathogenicity trials of the mites were inconclusive due to logistic problems. Currently we are attempting to develop a method to maintain the mites without bee hosts for a simplified pathogenicity assay

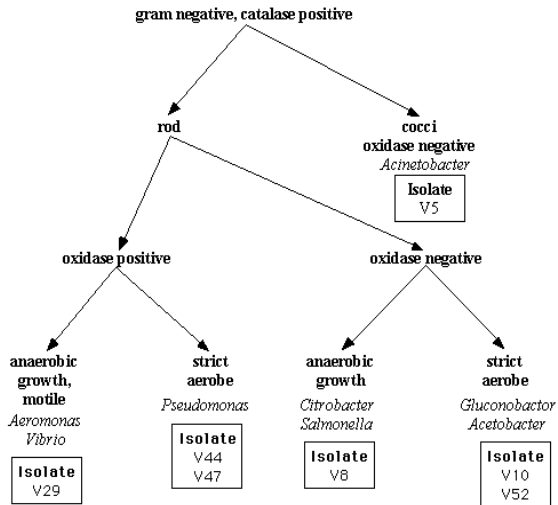


Figure 3. Test results used to determine possible genera of unknown gram negative isolates

## ACKNOWLEDGMENTS

I would like to thank the University of Wisconsin-La Crosse for the undergraduate research grant that allowed us to begin this project. I would also like to thank Dr. Bonnie Jo Bratina for her help, her ideas, her experience, her patience, and her friendship.

## REFERENCES

- Gerhardt, Philip, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips. 1981. p. 26-27, 413, 420. Manual of methods for general bacteriology. American Society for Microbiology, Washington DC
- Hartley, T. 1999. Fewer busy bees. Business First. 42(15):1-2.
- Johanowicz, D. L., M.A. Hoy. 1999. *Wolbachia* infection dynamics in experimental laboratory populations of *Metaseiulus occidentalis*. Entomologia Experimentalis et Applicata. 93:258-259.
- Madigan, Michael T., J.M. Martinko, J. Parker. 1997. p. 390-391, 724-726. Brock biology of microorganisms 8<sup>th</sup> Ed. Prentice Hall, Upper Saddle River, N.J.
- Morse, Roger A. 1994. The complete guide to beekeeping. Dutton/Plume, Santa Ana, CA.
- Morse, Roger. 1999. Detecting varroa. Bee Culture. 9(127):27-30.

