Modified Hanging Drop

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Introduction

Although recognizing visual signs of disease and in-field tests are often the first techniques used to diagnose foulbrood in honey bee colonies, sometimes greater diagnostic accuracy is required. In these cases, samples from suspect colonies can be collected and analyzed in a laboratory setting using a variety of techniques. One such technique is a relatively simple slide preparation method called the modified hanging drop. This technique allows for the diagnosis of both European foulbrood (EFB) and American foulbrood (AFB) through microscopic identification of the causative agents *Melissococcus plutonius* (EFB) and *Paenibacillus larvae* (AFB) from a single slide prepared from one symptomatic brood cell. This test can be very useful for distinguishing the foulbroods from other brood diseases with similar visual signs.

Choosing the Right Cells

For this test, it is important to choose cells that are exhibiting visual signs of either EFB or AFB. With EFB, look for larvae with an early infection, which are most likely to have the highest concentrations of live *M. plutonius*. If you suspect EFB, choose a larva that has not yet started to decompose, for example, one that is malpositioned (twisted or tipped over) or yellowed or that has started to lose internal pressure. For EFB, do not select a capped cell, scale, or larva that appears heavily decomposed. In late-stage infection, secondary bacteria take over and can be difficult to visualize the clusters of *M. plutonius* (Figure 1). If you are not sure what disease the colony is exhibiting, individually collect larvae exhibiting a variety of signs.

SUPPLIES NEEDED [in the field]

Brood frame exhibiting visible signs of AFB or EFB

Sample tubes

Collection tool (e.g., toothpick or lab spatula)

Figure 1: Visual progression of larvae with EFB. (1) An earlier stage of EFB and a great cell for this test. (2) A decent option for this test, although may sometimes provide a false negative result. (3) A very late stage of EFB. At this point, secondary bacteria likely have started to take over, which can cause a false negative result. Photos by Michigan State University.

With AFB, look for brood cells that are more likely to have spores. Spores are at their highest concentrations in late infection and are at lower numbers in early infection, so for AFB detection you will have the best luck choosing a black scale. While it is easy to see the vegetative state of *P. larvae* in earlier infections, there are other bacteria that can look similar, so it is hard to be certain without seeing spores.

Use a toothpick or other collection tool to collect suspect brood in a sterile sample tube. Take the sample to a lab for slide preparation. Samples can be transported at room temperature for this technique.

Preparing the Slide

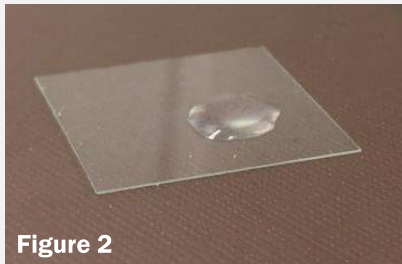
Figure 2

Start by placing a small drop of distilled water on a clean cover slip (Figure 2). Take a sterile swab and thoroughly macerate the sampled brood in the sample tube.

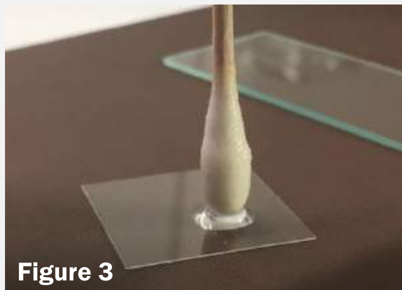
Figure 3

Gently touch the swab into the drop of water on the cover slip until the drop becomes slightly turbid (Figure 3). Do not transfer too much bacteria to the water because this will make the slide hard to read.

Figure 4

Hold the cover slip in self-closing forceps and place them under an infrared heat lamp to fix the sample to the slip (Figure 4).

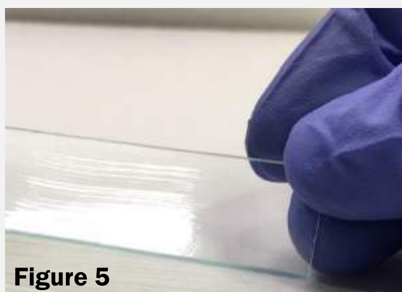
Figure 5

While the cover slip is fixing, label a microscope slide and gently spread immersion oil on the slide to form a thin layer (Figure 5).

Images Dr. Peter Fowler, Michigan State University.

SUPPLIES NEEDED [in the lab]

Nitrile gloves

Cover slip

Distilled water

Cotton swab

Self-closing forceps (or a tight clothespin)

Infrared heat lamp

Microscope slide

Type A immersion oil (Type B will not work)

Carbol fuschin (aka, Ziehl-Neelsen stain)
Note: this stain is hazardous to human health; ensure that you are following the directions on the Safety Data Sheet for this product.

Blotting paper

Compound light microscope with 100x oil immersion lens

Figure 6

Once the cover slip is fully heat fixed, flood the side containing the fixed sample with carbol fuschin stain for 30 seconds (Figure 6).

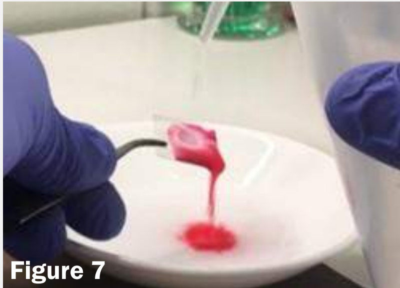
Figure 7

Gently rinse the slip with distilled water, leaving a little bit of water on the slip (Figure 7).

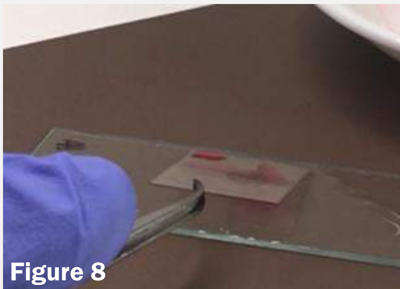
Figure 8

Carefully place the cover slip water/sample side down onto the thin layer of oil on the prepared microscope slide (Figure 8).

Carefully blot away any excess water with blotting paper.

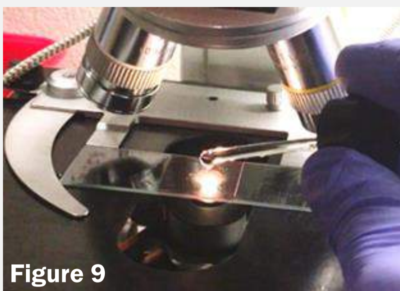
Figure 9

Place the slide on the microscope and rotate the objective lens so that there is open space above the slide (i.e., so that no lens is directly above the slide). Place a drop of immersion oil on top of the cover slip (Figure 9).

Adjust the microscope focus to observe the prepared sample.

Images Dr. Peter Fowler, Michigan State University.

Common Errors to Avoid

When macerating the larva or scale with the cotton swab it is helpful to wet the swab first in clean distilled water so it doesn't absorb the drop from the cover slip when transferring the sample.

When staining, the carbol fuchsin easily evaporates, so you may need to add additional stain to the slide to allow even staining for the 30 seconds required.

When rinsing, be sure to use a very light stream of distilled water and only rinse briefly, the water on the slide when you invert it may be slightly pink still from the stain. You want to be careful not to wash off all the non-adhered spores if you suspect AFB.

Identifying the Foulbroods

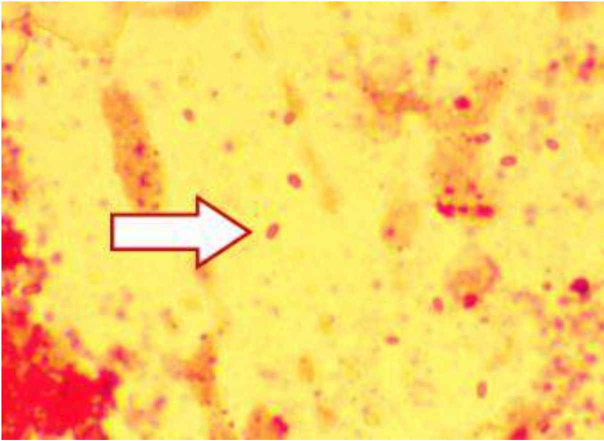

Figure 10: *P. larvae* spores under magnification. Photo by Sam Abban, USDA-ARS.

Adjust the microscope focus to observe the prepared sample. If *P. larvae* bacteria (AFB) are present, you will see AFB spores, which are small and oval-shaped and exhibit brownian motion (i.e., random motion of suspended particles). These spores are best seen at the water/oil interface of the slide (Figure 10).

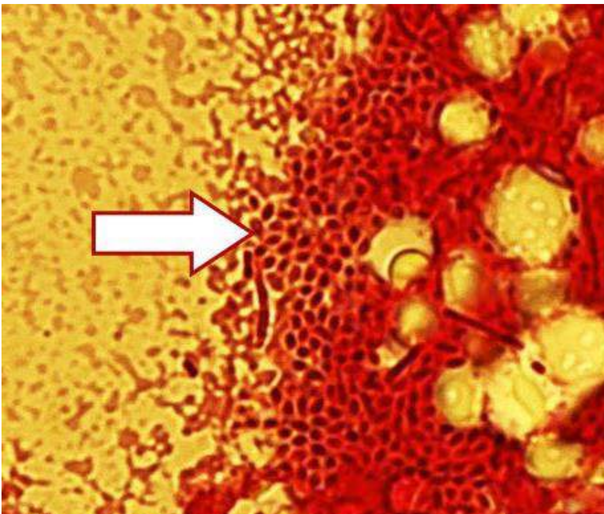

Figure 11: M. plutonius under magnification in evenly stained clusters. Photo by Sam Abban.

By contrast, *M. plutonius* bacteria (EFB) are also small and oval-shaped but tend to form in unmoving evenly stained clusters adhered to the cover slip (Figure 11). It is very common to see non-foulbrood objects on a slide prepared with the modified hanging drop technique. EFB bacteria are sometimes seen alongside a secondary invader called *P. alvei*, which are oval-shaped, but much larger than either EFB bacteria or AFB spores.

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