Honey bee diseases

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Part 1. Diagnostic overview

Summary

Four major honey bee diseases are recognised in Australia. These are American foulbrood (AFB), European foulbrood (EFB), nosemosis and chalkbrood. New Zealand has all of these diseases except EFB and also has varroosis, which has not been detected in Australia.

AFB, caused by the bacterium Paenibacillus larvae*, is a disease of older honey bee brood that is fatal to honey bee colonies once it becomes established. A diagnosis of AFB is best achieved by the microscopic examination of stained smears of dead or sick larvae. Ellipsoidal, spores 1.3 x 0.6 μm, are observed after staining with 0.2% carbol fuchsin. The culture of* P. larvae *from bulked honey samples can also be used to trace sources of AFB.*

EFB, caused by the bacterium Melissococcus plutonius*, is a disease of young honey bee brood. A diagnosis of EFB can be achieved by the microscopic examination of smears of dead or sick larvae. Smears stained with 0.2% carbol fuchsin reveal the causative organisms as lanceolate cocci 1.0–1.3 μm long by 0.5–0.7 μm wide. Secondary bacterial invaders, especially* Paenibacillus alvei*, are commonly found in infected larvae and may complicate the diagnosis.*

Nosemosis is caused by the microsporidia Nosema apis *or* Nosema ceranae*. Nosemosis associated with* N. apis *is characterised by spring dwindling of adult bee populations, decreased honey production and decreased brood production, and in severe cases may kill colonies. The effects of* N. ceranae *on honey bee colonies are unclear as it has only recently been detected in the European honey bee (*Apis mellifera*). Diagnosis of nosemosis is achieved by the microscopic examination of an aqueous suspension prepared from grinding the abdomens of about 20 old bees in a mortar with a pestle. The refractile spores are cylindrical with rounded ends and are 4.5 μm long by 2.4 μm wide. Differentiation between* N. apis *and* N. ceranae *can only be reliably achieved using molecular techniques such as PCR.*

Chalkbrood, a disease of older brood, is caused by the fungus Ascosphaera apis*. Diagnosis is achieved by microscopy of diseased brood that contains fungal spore cysts. These spore cysts, which are 60 μm in diameter, contain spore balls (12 μm diameter) that, in turn, contain spores (2.9 x 1.4 μm). A PCR is available for rapid, specific detection and identification of the fungus.*

Varroosis is caused by Varroa destructor*, an external parasitic mite that feeds on larvae and adult bees. The large oval-shaped red–brown female mites are 1.1 mm long and 1.6 mm wide, and are visible to the naked eye. Different strains of* V. destructor*, and all known genotypes of* Varroa *spp., are identified from their mtDNA CO-I gene sequences.*

Aetiology

American foulbrood

AFB is a disease of honey bee brood (larvae and pupae) caused by the bacterium *Paenibacillus larvae.*[1] It is a Gram-positive, rod-shaped bacterium 1.5–6 µm x 0.6 µm with motile peritrichous flagella that produce spores that are 2.0 x 0.8 µm. This species includes the previously known *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae.*[1] Genotyping of *P. larvae* isolates using enterobacterial repetitive intergenic consensus (ERIC)-PCR has identified four different ERIC patterns, designated ERIC 1–IV. Field isolates from bee hives showing the classical signs of AFB fall into one of either the ERIC 1 or ERIC II group. The reference strains for *P. larvae* subsp. *pulvifaciens* were determined to be ERIC pattern types III and IV.[1] There are no published reports of ERIC types in Australia although in a preliminary study only ERIC I was identified in Australian isolates of *P. larvae* (Hornitzky, unpublished).

European foulbrood

EFB is a disease of honey bee larvae caused by the bacterium *Melissococcus plutonius*.[2]

Nosemosis

Nosemosis is the most widespread of adult bee diseases and causes significant economic losses to beekeepers worldwide. This disease was originally thought to be caused by a single species, *Nosema apis*, a microsporidian which has a range of effects on honey bee colonies and adult bees. In 1994, a microsporidian *N. ceranae*, similar to *N. apis*, was described in Asian honey bees (*Apis cerana*).[3]

Chalkbrood

Chalkbrood is a disease of bee larvae caused by the fungus *Ascosphaera apis*. It was first reported in Australia in 1993 from south-east Queensland and by 1995 had spread to New South Wales, South Australia, Victoria, and Western Australia. Chalkbrood was found in New Zealand in 1984.

Varroosis

Varroosis is caused by the mite *Varroa destructor*. This mite has long been referred to as *V. jacobsoni*, but has now been shown to be a unique sibling species that has been concealed within the '*V. jacobsoni* species complex'.[4]

Clinical signs

American foulbrood

Infected brood generally dies after the cells have been capped over and the larvae have stretched out on their backs with the head towards the cell cappings (Figure 1). When the brood dies in the pupal stage the form of the pupa is carried through to the last stages of decay and the mouthparts are characteristically turned up toward the top side of the cell. The infected brood becomes slightly discoloured — light brown at first, then darker brown as the disease progresses with the body colour being even throughout. The larval body loses its segmentation.

After about 1 month larvae and pupae dry to form a dark scale that adheres to the wall of the cell and cannot be removed. The cappings over brood cells containing dead larvae or pupae sink inwards, become moist, and have a discoloured dark chocolate appearance. Some of these capped cells are punctured, the result of attempts by bees to remove the dead brood. Other cells will have their caps totally removed leaving the infective larval or pupal remains exposed. A probe such as a match-stick thrust into the remains at the sunken capped stage will, when removed, draw out the brown, semi-fluid remains in a ropy thread.

In heavily infected colonies, the brood pattern has a 'pepperpot' appearance due to the irregular arrangement of healthy cells intermingled with uncapped cells and capped cells of dead brood with punctured and sunken cappings. This 'pepperpot' appearance can easily be distinguished from that in EFB; in AFB the cappings have a dark chocolate appearance, whereas the cappings on dead brood of EFB are not significantly discoloured.[6]

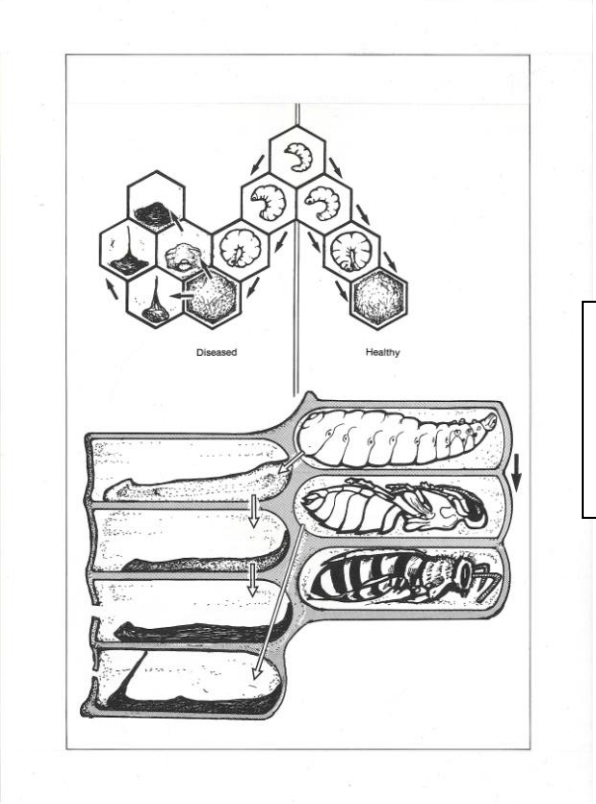

Figure 1. Stages in the development of American foulbrood compared with the normal development of the honey bee. [5] (Healthy on the right and diseased on the left.)

European foulbrood

The death of 4- or 5-day-old larvae is an indication of EFB. The larvae may collapse from their upright position and turn yellow–brown. They usually dry up to form a loosely attached brown scale. In some cases, there may be a small degree of ropiness if secondary infection with *P alvei* is present.

The smell of infected brood varies. The brood may give off a foul odour characteristic of cultures of *P alvei,* or a sour smell when *Enterococcus faecalis* is present. There is no

smell at all when these two secondary invaders are not present. Outer combs in the brood nest may show signs earlier and may have a heavier infection than inner combs.

Hives infected with EFB may show none of the above signs if nurse bees have ejected all diseased larvae before these characteristics become evident.

Diseased or dead larvae that have not decomposed may be dissected easily on a microscope slide by grasping the cuticle at the centre of a larva with two pairs of forceps which are then pulled apart. The midgut contents are left exposed on the slide within the transparent peritrophic membrane, which is partially or completely filled with bacteria in opaque chalk white clumps. The contents of normal midguts, which are less easily dissected, appear translucent and golden brown.[6]

Nosemosis

The effects of *N. apis* on the colony include dwindling of adult bee populations in spring, decreased honey production and decreased brood production, and in severe cases colonies may die.[6,7] *N. apis* reduces the lifespan of infected bees by about half in spring and summer.[8] Infected bees do not fully develop their hypopharyngeal glands, resulting in up to 15% of eggs in severely infected colonies failing to produce mature larvae in early summer.[9] Infected queens are generally superseded within a few weeks.[6,10]

Although infection with *N. apis* causes a syndrome of short duration, *N. ceranae* does not cause such a syndrome. Infection with *N. ceranae* has been observed only in association with non-specific symptoms, such as a gradual depopulation, higher autumn–winter colony deaths, or low honey production.[11]

Chalkbrood

Larvae die of chalkbrood usually after their cells have been capped. Among the first signs of infection are small perforations in otherwise normal cell cappings. When uncapped, dead larvae at first are somewhat white, swollen, fluffy and sponge-like, and may take on the hexagonal shape of the cell. Later they become hard and chalk-like in appearance, when they are called 'mummies', and will either remain white or, if the fungus develops spore cysts, turn grey or black. The mummies remain white if they are infected with only one mating strain of the fungus but will turn grey or black when infected with both mating strains of the fungus as a result of the production of spore cysts. Spores and vegetative stages of *A. apis* and other *Ascosphaera* spp. are commonly found in honey.

By the 'mummy' stage the cappings have frequently been removed by the bees. The beekeeper can remove cappings, with a match or twig, from diseased hives and usually find diseased brood at various stages of development.[12]

Varroosis

Adult female *V. destructor* usually occur inside sealed brood cells or partly hidden between abdominal segments of adults bees. Hence they are not easily noticed by beekeepers. Slightly infested colonies may show no clinical signs of infestation. Severely infested colonies show less vigour than non-infested colonies and are otherwise characterised by:

- irregular or 'spotty' brood patterns, caused by nurse bees removing infested or dead brood;
- disfigured and/or stunted adult bees, with deformed wings or legs;

- white debris on the walls or base of capped brood cells;
- pale or dark red spots on otherwise white bee pupae;
- bees discarding brood from the colony; and
- perforated cell cappings or uncapped cells.

Epidemiology

American foulbrood

Larvae become infected by swallowing bacterial spores, which germinate within 24 hours of entering the larval gut. Young larvae up to 24 hours old are most susceptible, while those more than two days old are immune.[13] The bacillus proliferates in the general tissue of larvae during the quiescent period before pupation, causing rapid death. Sporulation occurs 9 to 11 days after hatching, and 2500 million spores may form in one larva.[14] Secondary organisms are unable to grow in the remains of larvae probably because of an antibiotic released by *P. larvae*.[15]

There is no obvious seasonal pattern of disease and spores can remain viable for more than 33 years. Infection may be suppressed if larvae are removed before sporulation and this, coupled with the age susceptibility, makes the natural rate of spread low.[16]

Attempts by worker bees to remove diseased larvae may be effective, but may also spread infection around the hive and to the rest of the brood. The queen may also lay eggs in cells that have become contaminated. As larvae die, the hive becomes weaker and may eventually be abandoned ('die-out'). When food is scarce, bees may rob honey from weaker or abandoned infected colonies and introduce infection to their own colony.

The management practices of the apiarist also influence outbreaks of AFB. After honey harvesting, supers (boxes from which honey is collected) may be returned to hives other than the original hives, thus spreading infection throughout an apiary. Apiarists who do not use a 'barrier system' to restrict the spread of frames or supers to the parent hive are more likely to spread infection within an apiary.

European foulbrood

A balance can exist in an infected colony between the production and dissemination of *M. plutonius* and its elimination by nurse bees that, as well as feeding larvae with contaminated food, remove diseased individuals and clean out contaminated cells. Infection may persist in this way for many years with little or no obvious signs of disease.[6] Good nutrition appears to play a role in inhibiting disease outbreaks.

When disease outbreaks occur, infection usually stops spreading if the colony is strong enough to eject, rapidly, the many dead and diseased larvae. If this is not the case, the colonies may be destroyed, severely crippled, or sustain a chronic infection.

Larvae become infected by ingesting the bacteria with the food fed to them by nurse bees. Following ingestion by the larvae, *M. plutonius* divides rapidly almost filling the midgut and leading to one of four possible fates:[6]

- sudden death and ejection of larvae by nurse bees;
- secondary infection, particularly with *E. faecalis* and *P. alvei.*

- infected larvae may be sealed over but fail to pupate, being too weakened by infection (their remains may contain almost no organisms, but in the presence of the secondary invader *P. alvei*, their remains eventually become infected with masses of spores of this organism); or
- as *M. plutonius* merely competes with larvae for food, the larvae may survive and produce undersized adults if insufficient food is available.

Nosemosis

The spores of *N. apis* are ingested with the food of the bees or may be ingested while the bees are cleaning the hive before brood rearing commences. The spores germinate within 30 minutes and infest the epithelial cells of the ventriculus. The parasite develops and multiplies in the protoplasm of the host cells at 30^{o}C and the spores form after about 5 days. These are cast into the gut and pass to the rectum and are often still inside the host cell.[6]

In temperate climates, the proportion of individuals infected with *N. apis* in an undisturbed normal colony of honey bees reaches a peak in spring. The percentage then diminishes rapidly to a minimum shortly after mid-summer and does not increase again until late the following winter. This seasonal occurrence is due to the fact that infected honey bees do not defaecate in the hives during summer and thus the infection is not spread.[17]

It has also recently been shown that *N. ceranae* does not display the seasonality that is seen with *N. apis.* In a study of bee samples collected in Spain from 1999 to 2005, the typical *Nosema* seasonality was observed between 1999 and 2002, as characterised by an increase in infection levels especially in spring. However, from 2003 to 2005, this seasonality diminished and consistently high numbers of samples infected with *N. ceranae* were detected throughout 2005.[18] *N. ceranae* has also been demonstrated to cause significantly higher mortalities in laboratory experiments, indicating that it may be more virulent than *N. apis*.[42]

Chalkbrood

Larvae become infected by ingesting *A. apis* spores in their food. The spores germinate in the lumen of the gut where mycelia begin to grow, particularly at the hind end. The mycelia then penetrate the gut wall and eventually break out of the hind end of the larva's body, commonly leaving the head unaffected. When they occur, grey to black spore cysts ('fruiting bodies') form on the outside of the dead larvae.[12]

The spores of *A. apis* can remain infective for 15 years or more, although it has been reported that spores remain viable in pollen for one year and survive in honey for two years. Spores can be transmitted by infected queen bees, worker bees, sealed and unsealed brood from infected colonies, contaminated pollen, and contaminated tools.[19]

Clinical evidence of chalkbrood is most commonly seen in summer and early autumn, although *A. apis* can be present in hives all year. Chalkbrood has been reported to occur more frequently and at higher levels in colonies that are first weakened or stressed by intercurrent diseases, inclement weather (including high temperatures and humidity), poor ventilation, 'oversupering' (having too many boxes on the hive), low numbers of bees (especially nucleus colonies), nutritional deficiencies, and susceptible strains of bees.

Varroosis

Following the initial invasion of a bee colony by *V. destructor*, the colony will usually die within 2–4 years if left untreated. Colony death typically occurs when mite infestations become severe, usually during winter. Colonies that are restricted in their normal foraging activities become more susceptible to severe infestations, as do colonies that are kept in areas that provoke extended periods of brood-rearing when foraging conditions are poor. Feral honey bee colonies also become infested and may act as a reservoir of mites for non-infested, hived, colonies.

The spread of *V. destructor* between bee colonies is greatest in areas of high colony density. Mites can spread between colonies on drifting and robbing bees, and on infested 'escort' bees supplied in commercial queen cages. Mites can also be introduced to non-infested regions on natural swarms and when beekeepers move infested colonies.

Adult female *V. destructor* feed exclusively on the haemolymph ('blood') of honey bees. On adult honey bees, they feed after entering abdominal intersegmental spaces and piercing soft tissue with their mouth parts. On larval and pupal honey bees, the mites pierce the cuticle to access the haemolymph direct.

Female *V. destructor* breed on worker or drone honey bee pupae, although they prefer drone pupae. To breed, a mature female enters a bee brood cell containing a prepupa, just before the cell is capped. It immediately buries itself upside-down in the remaining bee food (jelly) at the bottom of the cell. About 5 hours later it emerges from the jelly onto the bee, pierces the bee cuticle, and feeds. Approximately 60 hours later the first egg, a male, is laid. Up to five more eggs, all female, are laid at 30 hour intervals. All the eggs will hatch into nymphal mites that, together with the mother mite, feed on the developing bee pupa. On worker bee pupae, only the nymphal males and 1–2 sister mites will develop into mature adults, whereas on drone pupae, the male and 1–3 sisters develop into mature adults. The mature male mite, which is smaller and paler than its nymphal sisters, subsequently mates with its sisters and dies within the cell. Consequently, all mites that are visible within a colony on adult bees are female. The newly-mated females and mother mite emerge with the callow bee (i.e. the emerged bee <4 days old) and are then free to parasitise other bee cells.

The weight of a bee pupa parasitised by *V. destructor* diminishes in proportion to the number of parasitising mites. Adult bees that have been parasitised as pupae begin to fly sooner and have shorter life-spans than bees not parasitised as pupae. The build-up and behaviour of mites within a colony also facilitates the spread of lethal viral infections among bees, particularly infections of deformed wing virus. Thus, heavy mite infestations can result in colony death.

Occurrence and Distribution

American foulbrood

AFB occurs in all major beekeeping areas worldwide.

European foulbrood

This condition has long been recognised in the northern hemisphere but the aetiological agent was only isolated in Australia in 1977.[20] It occurs in all Australian states except Western Australia. EFB is widely distributed throughout the main beekeeping areas of the world, but is not present in New Zealand.

Nosemosis

N. ceranae is widespread. It has been found in, Europe, Vietnam, the United Kingdom, the United States, South America and, more recently, in Australia.[21,22]

Chalkbrood

Chalkbrood is commonly found in most beekeeping countries.[12]

Varroosis

V. destructor is a parasite of honey bee colonies in Africa, the Americas, Asia, Europe (including the United Kingdom) and the Middle East. It was detected for the first time in New Zealand in April 2000.[23]

Diagnostic Tests (General)

American foulbrood

The laboratory diagnosis of AFB is based on the detection of *P. larvae* in diseased brood. This is best achieved by the microscopic examination of diseased individuals for spores of *P. larvae*. Alternatively, culture for *P. larvae* or PCR of diseased brood may be used for diagnosis. The culture of bulked honey samples is also a useful method to trace sources of infection.

European foulbrood

The laboratory diagnosis of EFB is based on the detection of *M. plutonius* in diseased brood. This is best achieved by the microscopic examination of diseased individuals for *M. plutonius.* PCR assays have been developed for the diagnosis of EFB and the detection of *M. plutonius* in bee products, but are not required for routine diagnostic purposes.[24,25,26]

Nosemosis

Microscopy of ground up bees or bee abdomens is sufficient for the diagnosis of nosemosis. PCR is required to differentiate between *N. apis* and *N. ceranae*.

Chalkbrood

The laboratory diagnosis of chalkbrood is based on the demonstration of the causative agent (*A. apis*) in diseased material. Alternatively, PCR can also be used for the confirmation of chalkbrood.

Varroosis

Members of *V. destructor* are distinguished from other varroa mites by their behavioural, morphological and genetic characteristics.[27]

Part 2 Test Methods

Collection of Specimens

American foulbrood

Ideally, smears of diseased brood can be prepared by placing three or four diseased larvae, that have stretched out on their backs, onto a glass microscope slide. The larvae are macerated and excess larval material is pushed off the edge of the slide.[28] After air-drying, the slide can be forwarded to the laboratory. Alternatively, a section of brood comb, of about 20 cm^2, containing diseased brood can be excised from the brood frame and submitted to the laboratory.

European foulbrood

The same procedure for the collection of specimens as described for AFB should also be used for EFB except that the larvae selected should be about 4 to 5 days old.

Nosemosis

Young (nurse) bees are unsuitable for the laboratory diagnosis of nosemosis. Bees collected from under the top lid, or from the outside of the brood nest, are old bees and suitable for nosemosis diagnosis.

Chalkbrood

Collect mummies from the entrance of hives, or from brood combs, or send a section of brood combs containing mummies.

Varroosis

Three types of specimens (colony debris, brood specimens and adult bees) are recommended for the diagnosis of varroosis.[29] These are recommended in countries where varroosis is known to occur. Australia has a technical response plan for varroosis in the AUSVETPLAN for Bee Diseases and Pests, and Exotic Bee Species, which is available from Animal Health Australia and should be consulted if varroosis is suspected.

Colony debris: Place an insert covered with mesh on the floor of the hive. If the insert is not covered with mesh, or smeared with grease, the bees will dispose of the mites outside of the hive. Within a few days the debris should be collected and submitted to the laboratory for diagnosis.

Brood specimens: Frames containing drone brood from suspect colonies should be sent to the laboratory.

Adult bees: Collect 200–250 bees from unsealed brood combs. Samples should be taken from both sides of at least three uncapped brood combs.

Transport of Specimens to the Laboratory

American foulbrood and European foulbrood

Microscope slides should be placed in slide containers or reinforced with cardboard before sending to the laboratory. Brood comb sections should be wrapped in paper and placed in

a cardboard box to ensure they are not crushed in transit. Comb sections should not be sent in plastic bags.

Nosemosis

Bees can be submitted live in small bee cages, dead, or fixed by placing them in 70% ethanol or methylated spirits. Live bees should be labelled as such on the package. The ethanol or methylated spirits should be drained from the containers prior to transport by posting or courier.

Chalkbrood

Brood comb sections should be wrapped in paper and placed in a cardboard box to ensure they are not crushed in transit. Comb sections should not be sent in plastic bags. Mummies can be submitted in 'Jiffy'® bags or small cardboard boxes.

Varroosis

Colony debris: The insert should be wrapped in paper and sent to the laboratory.

Brood specimens: Brood comb sections should be wrapped in paper and placed in a cardboard box to ensure they are not crushed in transit. Comb sections should not be sent in plastic bags.

Adult bees: Live bees of such large numbers are best delivered to the laboratory.[29]

Microscopy of brood or bees for the diagnosis of diseases

American foulbrood and European foulbrood

Diagnosis of AFB by microscopic examination of stained smears of dead or sick larvae in which *P. larvae* spores or *M. plutonius* vegetative cells are present, is entirely satisfactory.[28]

These air-dried smears submitted to the laboratory should be processed as follows:

(a) Heat-fix smears.

(b) Flood the slides with 0.2% carbol fuchsin for 30 seconds.

(c) Wash off the stain and allow to air dry or gently blot dry with absorbent material.
A diagnosis of AFB can be made when *P. larvae* spores, which are about 1.3 x 0.6 μm, ellipsoidal and thick-rimmed, are detected using microscopy (magnification x1000).[28,30]

A diagnosis of EFB can be confirmed when *M. plutonius* is present in large numbers in smears prepared from dead or sick larvae. The presence of many lanceolate cocci, 1.0–1.3 μm long and 0.5–0.7 μm wide, is reliable for a positive diagnosis. The presence of many cocci resembling those of *E. faecalis*, and/or spores resembling those of *P. alvei*, provides strong circumstantial evidence of EFB, and a search of a few more smears will usually reveal lanceolate cocci of *M. plutonius*.

P. alvei is a common secondary bacterial invader in larvae infected with *M. plutonius.* The spores of *P. alvei* are often confused with the spores of *P. larvae*. However, they can be distinguished from *P. larvae* spores as they are larger (about 2.0 μm x 0.8 μm versus about 1.3 μm x 0.6 μm) and stain more deeply with 0.2% carbol fuchsin. If further confirmation

of the identity of *P. alvei* is required, this organism can be cultured from stained, heat-fixed smears by placing a drop of sterile water on the slide and then culturing a suspension of water and larval material onto sheep blood agar without nalidixic acid. *P. alvei* will grow after overnight incubation of these plates and has a tendency to swarm even on dry plates. *P. larvae* will not grow after overnight incubation.[28]

Nosemosis

A reliable method of diagnosis of *N. apis* infection is the microscopic examination of infected bees for typical spores. This can be achieved by killing 20 bees (by storing them at -20^{o}C overnight) that have preferably been taken at random from the colony entrance. Their abdomens should then be snipped off at the waist and placed into a mortar, although whole bees can also be used. A few drops of water are added and the mass is ground up with a pestle. The mass is then pushed to one side and the mortar tilted to allow the fluid to drain to the other side. A drop of fluid is placed on a slide, covered with a glass cover slip and examined.

N. apis spores are easily identified after a little experience. They are short, cylindrical with rounded ends and about 4.5 μm x 2.4 μm. They have a much higher refractive index than the water in which they are usually examined, giving them a distinctive appearance. The only other bodies with which they are likely to be confused are the spores of some fungi and yeast cells. However, the latter are not usually highly refractive. *N. apis* spores can be found in enormous numbers, especially at the end of winter and the beginning of spring in severely affected colonies. If a quantitative assay is preferred, the method described by Cantwell [31] is recommended.

Chalkbrood

The laboratory diagnosis of chalkbrood is based on the demonstration of the causative agent (*A. apis*) in diseased material. This is achieved by mounting some diseased material, preferably 'mummies' that have turned grey or black, on a microscope slide, adding water or a dye, and mixing thoroughly. The resultant suspension is then examined under the microscope. The presence of spore cysts is usually sufficient to make a diagnosis. These spore cysts, which are about 60 μm in diameter, contain smaller round bodies known as spore balls (average 12 μm in diameter) in which the spores (average 2.9 x 1.4 μm), the most infective stage of the fungus, are found.[19]

In samples in which only white 'mummies' have been submitted, and spore-producing bodies cannot be detected when examined under the microscope, it may be necessary to grow the fungus on potato-dextrose agar or yeast-glucose-phosphate medium.

Culture of *P. larvae* from bulk honey for detection of American foulbrood

The culture of bulked honey samples (obtained by extracting honey from multiple colonies) for *P. larvae* spores, and trace back to hives from which infected honey was extracted, can be an effective means of detecting AFB outbreaks. A broad range of culture media and methods for processing honey has been developed[30] but the following method is recommended.

Mix 75 mL, of the bulk honey sample with 75 mL of phosphate buffered saline (PBS pH 7.2). Smaller volumes of honey can also be used. Centrifuge the samples for 45 minutes at

3000 ***g***. Pour off the supernatant, leaving about 1.5 mL of fluid that is used to mix with the sediment in the bottles. Place 0.5 mL in a 5 mL glass bottle and heat at 80°C for 15 minutes. Streak a loop-full of the sample onto a sheep blood agar plate comprising Blood Agar Base No. 2 (Oxoid) supplemented with 7% citrated ovine blood and 3 μg/mL nalidixic acid (SBANa). The role of nalidixic acid is to inhibit the growth of *P. alvei* which is commonly found in honey in areas where EFB is endemic.

Colonies appear in 2–4 days. After 4 days on SBA, colonies are flat, grey, approximately 6 mm in diameter and have an irregular edge. Gram-stained smears of these colonies show Gram-positive rods that are 0.5-0.6 μm wide and 1.5-6.0 μm long. *P. larvae* will sporulate successfully on SBANa plates.

A quick and simple test for the confirmation of the identity of *P. larvae,* once the colony morphology and microscopic criteria have been fulfilled, is a negative catalase test. Catalase-positive strains of *P. larvae* are unknown; most other aerobic spore-forming bacteria are catalase-positive.[32]

Record the number of colony forming units cultured from the honey samples as '+' if 1–20 colonies develop, '++' if 21–50 colonies develop, and '+++' if 50 or more colonies develop.[9]

The higher the concentration of spores in the sample, the more likely it is that AFB is present in the hives or there is a recent history of the disease. In an examination of 505 bulk honey samples in New South Wales, six (100%) of '+++', 11 (78.6%) of '++' and 22 (56.4%) of '+' honey samples were from diseased hives or those with recent histories of the disease.[33]

The culture of honey samples can also be used to monitor for the presence of *P. larvae* spores and is an effective tool in determining the prevalence of this organism in beekeeping areas.[33]

AFB spores detected in honey samples may also be unrelated to AFB-diseased hives. Spores may find their way into hives by robber bees or drifting bees. These hives may subsequently have spores in their honey, which may be extracted and detected without these hives showing signs of AFB. The use of shared extracting facilities, used drums containing honey, or transfer of honey from one beekeeper to another may also complicate traceback efforts. Hive treatment with oxytetracyline hydrochloride may mask signs of AFB for up to 14 months after treatment in infected hives.[33] Hence, honey from apparently normal hives may contain *P. larvae* spores that may be detected by this cultural procedure.

Culture of *A. apis* for diagnosis of chalkbrood

Yeast-glucose-phosphate agar is composed of 1% yeast extract (Oxoid), 0.1% cysteine or cystine (BDH), 1% glucose (Ajax), 1.35% KH2P04 (Ajax) and 1% soluble starch (Ajax).[35] The plates should be incubated at 37°C in an atmosphere containing 5-10% $C0_2$. Fungal colonies grow moderately slowly and are 5–7 cm in diameter after 10 days. They produce aerial mycelia that are deeply floccose or matted, white to pale buff, and may become coral to pale red–brown with age.[36] To confirm the identity as *A. apis* it is necessary to mate the isolate with one of two known mating strains of *A. apis.*[37] This is achieved by inoculating the culture medium with the suspect *A. apis* culture and inoculating the two

known mating strains on either side of the test isolate about 1 cm away. The fungus is confirmed as *A. apis* if it mates with one of the two mating strains by producing spore cysts. These appear as a brown line at the junction where the test fungus and control fungus meet and mate. Mating usually takes from 5 to 10 days; however, the production of spore balls, and the spores within, may take up to 15 days.

Molecular techniques for the detection of honey bee pathogens

American foulbrood

PCR assays have been developed for the diagnosis of AFB in diseased brood[38] and for the detection of *P. larvae* in honey.[39] However, the relatively inexpensive and quick diagnosis of AFB using microscopy and the quantitative feature of bacterial culture for honey samples is usually preferred.

European foulbrood

The PCR described by McKee et al.[24] can be used to identify *M. plutonius* in bee brood, bee products and suspect *M. plutonius* cultures.

PCR can be done on suspicious bacterial colonies transferred and grown in liquid medium.[25] Genomic DNA is prepared according to standard methods.[39] The DNA pellet is resuspended in 50 μL of 1 x TE buffer (10 mM Tris/HCl, pH 7.5; 1 nM ethylene diamine tetra-acetic acid (EDTA). Approximately 1–3 μg of genomic DNA is amplified in a 50 μL reaction. The PCR reaction can also be done with larvae. Each larva is incubated individually in liquid medium overnight at 30°C in an anaerobic jar containing hydrogen plus 10% CO_2.

The 50 μL reaction mixture contains 5–30 ng genomic DNA, 3 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphospate, 100 ng of the primers MP1 and MP2 (Table 1) of 10 x PCR buffer (100 mM Tris/HCl. pH 8.3; 15 nM $MgCl_2$; 500 mM KCl) and 1 U of *Taq* polymerase. Conditions of amplification consist of an initial denaturation cycle of 95°C for 2 minutes followed by 40 cycles of denaturation (95°C, 30 sec), primer annealing (61°C, 15 seconds) and primer extension 72°C, 1 minute) followed by an additional extension step of 5 minutes at 72°C. The third primer MP3 (Table 1) is used in conjunction with MP1 to amplify a DNA fragment from 1 μL of the primary PCR product obtained in the previous reaction. PCR conditions for the hemi-nested PCR are exactly as described above except the $MgCl_2$ concentration is lowered to 1.5 mM and the annealing temperature to 56°C.

Table 1. Primer sets to identify *M. plutonius*[24]

Reference	Primer	Sequence	Size
Govan	EFB-F	5'-GAAGAGGAGTTAAAAGGCGC-3'	812 bp
	EFB-R	5'TTATCTCTAAGGCGTTCAAAGG-3'	
Djordjevic/Mckee	MP1	5'-CTTTGAACGCCTTAGAGA-3'	486 bp
	MP2	5'-ATCATCTGTCCCACCTTA-3'	
	MP3	5'-TTAACCTCGCGGTCTTGCGTCTCTC-3'	276 bp

The molecular weights of the PCR products are determined by electrophoresis in a 1.0–1.5% agarose gel and staining with ethidium bromide.

Nosemosis

Several PCR assays have been developed for the differentiation of *N. apis* from *N. ceranae.* The method described by Martin-Hernandez et al. [18] is recommended.

The abdomens of 10–20 adult honey bees from each sample are macerated in 10 mL of distilled water. The suspension is then filtered and centrifuged at 800 ***g*** for 6 minutes. For DNA extraction, spore germination of the pellet is induced with 200 µL of freshly prepared germination buffer (0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH to 6.0 with orthophosphoric acid), and the mixture is incubated at 37°C for 15 minutes. The DNA extraction can be carried out using routine procedures or commercial kits.

The multiplex PCR can distinguish between *N. apis* and *N. ceranae* in a single reaction. PCR reactions are performed in 50 µL volumes containing 5 µL of template DNA, 25 µM of High Fidelity PCR Master Mix® (catalogue no. 12140314001; Roche Diagnostic), 0.4 µM of each primer (Table 2), 0.4 mM of each deoxynucleoside triphosphate, 3 mM Cl_2Mg, 0.2 mg/mL bovine serum albumin, 0.1% Triton X-100, and 5 µL of *N. apis* or *N. ceranae* DNA template. The parameters for amplification are: initial PCR activation step of 2 minutes at 94°C, 30 seconds at 61.8°C, and 50 seconds at 72°C plus a 5 second elongation cycle for each successive cycle and a final extension step at 72°C for 7 minutes. Negative controls (from DNA extraction) are included in all PCR procedures.

The molecular weights of PCR products are determined by electrophoresis in a 2% agarose TAE (Tris-acetate-ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with ethidium bromide and visualised using UV illumination.[18,41]

Table 2. Primers for the detection of *N. ceranae* and *N. apis*[18]

Specificity	Primer	Sequence[a]	Size
N. ceranae	218MITOC FOR	5'-CGGCGACGATGTGATATGAAA-ATATTAA-3'	218-219[b]
	218MITOC REV	5'-CCCGGTCATTCTCAAACAAAA-AACCG-3'	
N. apis	321APIS FOR	5'-GGGGGCATGTCTTTGACGTACTATGTA-3'	321
	321APIS REV	5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3'	

[a] CG tails added to primers are underlined

[b] There is a 1-bp difference in the amplicon size of *N. ceranae* depending on the sequences for *N. ceranae* available in GenBank (http:/www.ncbi.nlm.nih.gov).

Chalkbrood

Rapid detection and identification of *A. apis* can by performed by PCR using primers specific for *A. apis*.

DNA is extracted from the suspect fungus by either a rapid or slow DNA extraction method. For the rapid method, fungal spores or mycelia are placed in a PCR tube (a 0.5 mL Eppendorf tube) with an equal volume of glass beads and 100 µL distilled water. This mixture is vortexed for 30 seconds and centrifuged for 2 minutes at 6000 ***g*** in an

Eppendorf bench centrifuge. The supernatant is then removed to a clean PCR tube and used direct in the PCR reaction. For the slow extraction method, which produces cleaner DNA preparations than does the rapid method, 0.4–0.5 g of compressed fungus mycelia are ground in liquid nitrogen to a fine powder in a pre-cooled mortar and pestle. Three mL of freshly prepared K buffer (2.0 mg/mL proteinase K, 0.1 M Tris-HC1 pH 8.5, 0.05 M EDTA, 0.2 M NaCl and 1% SDS) is added, the mixture held in a water bath at 65^{o}C for one hour, then extracted with an equal volume of phenol saturated with 10 mM Tris-HCl, and 1 mM EDTA pH 8 (TE) at room temperature for 15 minutes. The phases are then separated by centrifugation at 2000 ***g*** for 10 minutes, the supernatant removed to a clean centrifuge tube, extracted as above with an equal volume of chloroform-isoamyl alcohol (24:1), and centrifuged to separate the phases. The supernatant is once again removed to a clean centrifuge tube where two volumes of cold ethanol are added to precipitate the DNA. The DNA is then pelleted by centrifuging at 6000 ***g*** for 10–15 minutes at 4^{o}C, resuspended in 75% ethanol, pelleted again at 6000 ***g*** for 5–10 minutes, dried at room temperature, resuspended in 500 μL TE containing 10 pg/mL RNase A and incubated at 37^{o}C for 30 minutes. This solution is extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) as described above and centrifuged to separate the phases. The supernatant is removed to a clean centrifuge tube, 50 μL of 3 M sodium acetate pH 6.0 added, and the DNA precipitated with 2.5 volumes of cold ethanol. The precipitated DNA is pelleted, resuspended and dried as described above, resuspended in 200 μL of distilled H_2O, and used direct in the PCR reaction or frozen at -20^{o}C until needed.

The nuclear rDNA region containing the sequence of the *A. apis* internal transcribed spacer regions and 5.8S rDNA (ITS1-5.8S-ITS2) is amplified by PCR using either one or two forward primer 5'-GCTAGGTGCCCCTAAACAAGGC- Y(CBP1) or 5'-TTTGAGTTCCCCCCTGGCTAGC- 3' (CBP2) in conjunction with the reverse primer 5'-ACTAGAGCGAAAGACAAAGCC- 3' (CBP3) using methods described by Anderson et al.[37] These primers were constructed from original sequence data generated by Anderson et al.[37] and are *A. apis*-specific. Each primer combination will generate a single PCR product of about 500 base pairs but the combination of CBP1 and CBP3 primers will generate a slightly larger product than the CBP2 and CBP3 primer combination. For PCR reactions, 2–20 ng of purified DNA are added to 5 μL of 0.01 mM AB28 primer, 5.0 μL 0.01 mM TW81 primer, 5.0 μL 10 x PCR buffer (670 mM Tris- HQ pH 8.8, 166 mM ammonium sulphate, 2 mg/mL gelatin, 15 MM M9C12 and 4.5% Triton X-100), 50 μM each of dATP, dCTP, dGTP and dTTP and one drop of oil. DNA is amplified using an automated thermal cycler and the following protocol: five minutes initial denaturation at 94^{o}C, after which 2 U of Taq polymerase is added; then 30 cycles of one minute at 94^{o}C, 1.5 minutes annealing at 54 to 55^{o}C, and two minutes extension at 72^{o}C. A final extension period of five minutes at 72^{o}C completes the amplification. Five μL of PCR product are then added to 2 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll [Type 400, Pharmacia]) in water, and electrophoresed together with Lamda DNA-Hind 111 as a marker in a 1% agarose gel containing 10 μg ethidium bromide per 100 mL TAE at 60 v for three hours. DNA bands are then visualised using an ultra-violet transilluminator.

Detection and identification of *Varroa* mites by mtDNA sequencing

Colony debris: Dry the debris for 24 hours. Flood the debris with ethanol. Stir continuously for 1 minute or, if the debris contains wax or propolis, stir for 10–20 minutes. Identify the mites that float to the surface.

Brood specimens: Remove the cappings of the brood cells with a knife or other suitable implement. Wash the brood cells direct into a sieve system with warm water from a hand-held shower. Collect the mites in the lower fine sieve (mesh width 1 mm) while the brood is gathered in the upper coarse sieve (mesh width 2–3 mm). Place the contents of the sieve on a light box where the mites can be identified.

Adult bees: Kill the bees by submersion in alcohol. Stir the container for 10 minutes. Separate the bees from the mites by means of a sieve with a mesh size of approximately 2–3 mm.

Identification of *V. destructor*: Members of *V. destructor* are distinguished from other varroa mites by their behavioural, morphological and genetic characteristics. There are currently six recognised strains (or haplotypes) of *V. destructor* designated as the Japan/Thailand, Korea, China, Vietnam, Nepal and Sri Lanka strains. Each can only be reliably distinguished by differences in their mitochondrial DNA (mtDNA) gene sequences for cytochrome oxidase I (CO-I).[27] Only two strains of varoa mites (the Korea and Japan/Thailand strains of *V. destructor*) are able to reproduce on *A. mellifera*. The Korea and Japan/Thailand strains of *V. destructor* are large (1.1 mm in length x 1.7 mm in width), and can be seen with the naked eye. They are easily recognised by their flattened oval shape and reddish brown colour.

Other varroa mites, such as *V. jacobsoni*, which are parasites of particular strains of Asian honey bees (*Apis cerana*) throughout Asia, are capable of invading *A. mellifera* colonies and entering susceptible brood cells. However, they lack the ability to produce eggs or offspring on *A. mellifera* brood.

To obtain DNA preparations for sequencing, individual female varroa are washed twice in 70% ethanol and placed in a small watch glass containing 40 µL of 2x lysis buffer (120 µg/mL proteinase K, 0.1 M KCl, 0.02 M Tris-HCl pH 8.3, 5 mM $MgCl_2$, 0.9% Tween 20, 0.9% NP40 and 0.02% gelatin). Then, with the aid of a dissecting microscope, tissue is dissected from each of the mites' legs. Lysis buffer containing the dissected tissue is transferred to a microtube, incubated at 65^{o}C for 30 minutes then at 95–100^{o}C for 10 minutes, diluted with 40 µL dH_2O, and 2–20 µL used as template in the PCR.

A 458 base pair region of the mtDNA CO-I gene is amplified by PCR from the DNA preparations from individual mites using the forward primer 5'-GG(A/G)GG(A/T)GA(C/T)CC(A/T)ATT(C/T)T(A/T)TATCAAC- 3' (COXF) and the reverse primer 5' -CCTGT(A/T)A(A/T)AATAGCAAATAC- 3' (COXRa), and following the PCR methods as described for *A. apis*.

Amplified DNA is then sequenced direct or after being cloned into a suitable vector (such as the pBlueScript SK vector). Sequencing is carried out using a conventional sequencing system, such as a Model 373A DNA Sequencing System® (Applied Biosystems), according to the manufacturer's instructions. The identity of the varroa DNA sequences is obtained by comparing the sequences obtained with varroa mtDNA CO-I gene sequences

that have been deposited in the GenBank database under the accession numbers AF106893 to AF106910. The GenBank accession numbers of the common Korea and less common Japan/Thailand strain of *V. destructor* are AF106899 and AF106897, respectively.[27]

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