

Honeybee diseases

MA Hornitzky

NSW Agriculture,
Elizabeth Macarthur Agriculture Institute,
PMB 8, CAMDEN, NSW 2570
michael.hornitzky@agric.nsw.gov.au

DL Anderson

CSIRO Entomology,
GPO Box 1700,
CANBERRA, ACT 2601
denis.anderson@ento.csiro.au

SUMMARY

Five major honeybee diseases are recognised in Australia. These are American foulbrood, European foulbrood, *Nosema apis* infection, Sacbrood and Chalkbrood. All these diseases except for European foulbrood, and also the *Varroa* mite, have been identified in New Zealand.

Identification of the agents

(a) American foulbrood – The causative agent, *Paenibacillus larvae* subsp. *larvae*, is detected by the microscopic examination of carbol fuchsin stained smears of dead or sick larvae. Larval remains are pulped and a thin smear made on a microscope slide. Ellipsoidal, thick rimmed spores, 1.3 x 0.6 mm, are observed after staining with 0.2% carbol fuchsin.

(b) European foulbrood - Microscopic examination of smears of pulped sick larvae, stained with 0.2% carbol fuchsin, reveals the causative organism, *Melissococcus pluton*, as lanceolate cocci, 1.0 to 1.3 mm long by 0.5 to 0.7 mm wide.

(c) *Nosema apis* infection – Infection is caused by the sporeforming microsporidian *Nosema apis* and is detected by the microscopic examination of an aqueous suspension prepared from grinding the abdomens of about 20 bees in a mortar and pestle. The refractile spores are cylindrical with

rounded ends and are 4.5 mm long by 2.4 mm wide.

(d) Sacbrood - An agar gel immunodiffusion test employing specific antiserum is used to detect viral antigen in extracts prepared from diseased larvae. The virus may be visualised by electron microscopy using the extracts used for immunodiffusion.

(e) Chalkbrood – The causative fungus, *Ascosphaera apis*, is detected by microscopic examination of an aqueous suspension of diseased larvae. The fungal spore cysts, 60 mm in diameter, contain spore balls (12 mm diameter) which, in turn, contain spores (2.9 x 1.4 mm). A polymerase chain reaction is available for rapid, specific detection and identification of the fungus.

(f) *Varroa* – *Varroa destructor* is an external parasitic mite that feeds externally on larvae and adult bees. The large oval-shaped reddish 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* are identified from their mtDNA CO-I gene sequences.

Serological Tests

There are no standard serological tests available for the above diseases.

Introduction

The following sections deal with the pathogenesis, epidemiology, clinical signs and laboratory diagnosis of the six major honeybee diseases that occur in Australia and/or New Zealand. These diseases can affect one or more of the following six stages in the development of the adult worker bee or the emerged adult bee:

- (a) The embryo develops for 3 days in the egg.
- (b) When the larva hatches from the egg, it is fed continuously for the next 5 days, while it is growing in an open cell, by young adult bees or 'nurse bees'.
- (c) On day 8 the fully-grown larva is sealed in its cell by nurse bees and then spins a cocoon. About 2 days after it is sealed over, the larva lies on its back with its head towards the cell capping.
- (d) The quiescent larva changes within a loosened fifth skin to a propupa, and after 2 days of this phase it sheds the fifth skin to become a white pupa.
- (e) The pupa, now resembling an adult bee in shape, slowly darkens in colour, beginning with the eyes.
- (f) The pupa sheds its skin, and a few hours later (21 days after the egg is laid) the adult emerges from its cell.¹

American Foulbrood

American foulbrood (AFB), also known as American brood disease, is a disease of honeybee brood, that is, larvae and pupae, caused by the bacterium *Paenibacillus larvae* subsp. *larvae*. It occurs in the temperate or subtropical regions of most continents and in New Zealand, Hawaii and some of the West Indies. In Australia it has been found in all States.²

Pathogenesis

Larvae become infected by swallowing bacterial spores, which germinate within 24 h of entering the larval gut. Young larvae up to 24 h old are most susceptible, while those over 2 days old are immune.³

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.⁴ Secondary organisms are unable to grow in the remains of larvae probably because of an antibiotic released by *P l larvae*.⁵

Epidemiology

Larvae become infected by spores spread by adult bees clearing away remains or by spores remaining in brood cells. There is no obvious seasonal pattern of disease signs 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.²

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 in cells that have become contaminated. As larvae die, the hive becomes weaker and may eventually be abandoned ('die-out'). In times when food is scarce, bees may rob honey from weaker and abandoned, infected hives and introduce infection to their own hive.

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 the 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.

Clinical Signs

The brood generally dies after the cells have been capped over and they have stretched out on their backs with the head towards the cell cappings. 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 brownish at first, then darker brown as the disease progresses with the body colouration being even throughout. The larval body loses its segmentation. After 1 month they dry to a very dark scale, which 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

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these capped cells are punctured, the result of attempts by bees to remove the dead larvae or pupae. Other cells will have their caps totally removed leaving the infective larval or pupal remains exposed. If a match-stick is thrust into the larval remains at the sunken capped stage and is then removed, it draws 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 European foulbrood because in AFB the cappings have a dark chocolate appearance whereas the cappings on dead brood of European foulbrood are not significantly discoloured.

Laboratory Diagnosis

Diagnosis of AFB by microscopic examination of stained smears of dead or sick larvae in which *P l larvae* spores are present is entirely satisfactory.⁶

Smears should be prepared as described in Appendix 1.

The culture of bulk honey samples for *P l larvae* spores and traceback to hives from which infected honey was extracted can be an effective means of detecting AFB outbreaks (Appendix 2). The culture of honey samples can also be used as a monitoring system for the presence of *P l larvae* spores and is an effective means of determining the prevalence of this organism in beekeeping areas.⁷

The higher the concentration of spores in the sample the more likely it is that the beekeeper has AFB in the hives or a recent history of the disease. In an examination of 505 bulk honey samples in New South Wales, 6 (100%) of '+++', 11 (78.6%) of '++' and 22 (56.4%) of '+' honey samples were from beekeepers with diseased hives or recent histories of disease.⁷

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 disease signs. The use of shared extracting facilities, used drums containing honey or transfer of honey from one beekeeper to

another may also complicate traceback efforts. Alternatively, hive treatment with oxytetracycline hydrochloride may mask disease signs for up to 14 months after treatment in infected hives.⁸ Hence, apparently normal hives may contain *P l larvae* spores in honey, which may be detected by this cultural procedure.

European Foulbrood

European foulbrood (EFB), also known as European brood disease, is a disease of honey bee larvae caused by the bacterium *Melissococcus pluton*.⁹ This condition has long been recognised in the northern hemisphere but its aetiological agent was only isolated in Australia in 1977.¹⁰ It occurs in all States except Western Australia. It is also not known to occur in New Zealand.

Pathogenesis

Larvae become infected by ingesting the bacteria with the food fed to them by nurse bees. Following ingestion by the larvae, *M pluton* divides rapidly almost filling the midgut and leading to one of four possible fates:¹

- (a) Sudden death and ejection of larvae by nurse bees.
- (b) Secondary infection particularly with *Enterococcus faecalis* and *Paenibacillus alvei*.
- (c) 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 event of the presence of the secondary invader *P alvei* their remains eventually become infected with masses of spores of this organism.
- (d) As *M pluton* merely competes with larvae for food, the larvae may survive and produce undersized adults if insufficient food is available.

Epidemiology

A balance can exist in an infected colony between the production and dissemination of *M pluton* and its elimination by nurse bees who, 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.¹ Good nutrition appears to play a role in inhibiting disease outbreaks.

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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.

Clinical Signs

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

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.

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 *E 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.¹

Laboratory Diagnosis

The diagnosis of EFB can be confirmed by microscopic examination of 0.2% carbol fuchsin stained smears of dead or sick larvae in which *M pluton* is present in large numbers in the midgut. The presence of many lanceolate cocci, 1.0 – 1.3 µm long x 0.5 – 0.7 µm wide, is reliable for a positive diagnosis. Many cocci resembling those of *E faecalis* and /or spores resembling those of *P l larvae* are very strong circumstantial evidence of EFB, and a further search of a few more smears will usually reveal lanceolate cocci of *M pluton*.

Smears should be prepared as described for AFB; however, the larvae sampled should be 3 or 4 days old.

P alvei is a common secondary bacterial invader in larvae infected with *M pluton*. The spores of *P alvei* are often confused with *P l larvae* spores. They can, however, be distinguished from *P l larvae* spores as they are larger (about 2.0 µm x 0.8 µm) and stain more deeply with 0.2% carbol fuchsin than *P l larvae* spores, which are about 1.3 µm x 0.6 µm.

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 l larvae* will not grow after overnight incubation.

Nosema Apis Infection

Nosema apis is a spore-forming microsporidian. The infection caused by this organism is by far the most widespread of adult bee diseases and is almost universal.

Pathogenesis

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 min and infest the epithelial cells of the ventriculus. The parasite develops and multiplies in the protoplasm of the host cells at 30°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.¹

Epidemiology

In temperate climates the percentage 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 honeybees do not

defecate in the hives during summer and thus the infection is not spread.¹¹

Clinical Signs

These signs are often confused with other troublesome conditions of adult bees, such as bee paralysis, starvation, chilling, pesticide poisoning and dysentery. The following signs may be observed:

- Disjointed wings, distended abdomens and the absence of the stinging reflex.
- Bees crawling about in the hive and in grass in the front of the hive.
- In cases of heavy infection, the honey bee ventriculus is enlarged, soft in consistency and white.
- Eggs (15%) in infected colonies may fail to produce mature larvae, compared with 1% in healthy colonies.
- Infected bees live only half as long as non-infected bees.¹²

Laboratory Diagnosis

The only reliable method of diagnosis of *N apis* infection is a microscopic examination of infected bees for typical spores. This can be achieved by killing 20 bees 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. 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 this fluid is placed on a slide, covered with a glass coverslip and examined.

N apis spores are easily identified after a little experience. They are short, cylindrical with rounded ends and are about 4.5 µm long x 2.4 µm wide. 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, these 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.

Sacbrood

Sacbrood is a disease of honeybee larvae caused by an isometric 28 nm virus. This infection has been frequently reported from New Zealand, Britain, Switzerland, Germany and North America. In Australia it has been recognised more frequently since 1976.

Pathogenesis

One of the characteristics of sacbrood is the accumulation of ecdysial fluid around the pupal integument. This fluid is rich in sacbrood virus.

Larvae with this disease are found mostly in spring and early summer.

Although infection of adult bees in the laboratory has little effect on their life span, sacbrood develops quickly following infection of young larvae. Larvae 12 – 36 h old, which are inoculated with sacbrood virus by mouth, show signs in 48 h and die shortly after. Electron microscopy reveals the sacbrood virus to be abundant in the cytoplasm of fat and muscle cells and tracheal end-cells in larvae, both those that exhibit the signs and those that do not.¹³

Clinical Signs

Partially uncapped cells may be scattered among the capped brood. Capped cells, which remain after the surrounding brood has emerged, may also be observed.

Partially capped or uncapped cells contain individuals, which have died when they have spun their cocoons and stretched out to pupate. Most die in the prepupal state.

Their last larval (prepupal) skin loosens and becomes separated from the pupal skin below a layer of fluid as usual, but is not shed. An unusual amount of fluid accumulates between the skins before the prepupa dies, causing a flabby sac-like condition in which the larva appears to be in a gondola shape. The body segmentation is retained.

Before the prepupa dies the underlying cuticle darkens, beginning at the eyes and spreading posteriorly. This premature darkening appears first as a light pale yellow, then light brown and finally dark brown.

The 'matchstick test' (see AFB) is always negative. However, the remains may 'rope' for 10 – 12 mm under certain circumstances but the body tissues are not evenly coloured and of a

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smooth consistency as seen with AFB. The thread is not elastic and does not 'snap back' as with AFB.

If they are not thrown out of the cells, these dark brown individuals dry and become wrinkled with brittle scales, which, unlike the scales of AFB, are easily removed from the cells.

There is no distinctive odour associated with sacbrood

Laboratory Diagnosis

The laboratory diagnosis of this disease requires the use of an agar gel immunodiffusion (AGID) test using specific antiserum to detect viral antigen. Electron microscopy can be used to confirm the diagnosis made by the AGID test. Preparation of virus extracts and antiserum are described in Appendices 3 and 4.

Chalkbrood

Chalkbrood is a disease of 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, Victoria and South Australia. Chalkbrood was found in New Zealand in 1984. It is common in most beekeeping countries.¹⁴

Pathogenesis

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.¹⁴

Epidemiology

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 1 year and survive in honey for 2 years. Spores can be transmitted by infected queen bees, worker bees, sealed and unsealed brood from infected colonies, contaminated pollen and contaminated tools of the beekeeper.¹⁵

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.

Clinical signs

Larvae die of chalkbrood usually after their cells have been capped. One of the first signs is small perforations in otherwise normal cell cappings. When uncapped, dead larvae at first are somewhat fluffy white, swollen and sponge-like and may take on the hexagonal shape of the cell. Later they become hard and chalk-like in appearance and are called "mummies" which will either remain whitish or, if the fungus develops spore cysts, turn grey or black. The mummies remain whitish 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, in diseased hives and usually find diseased brood at various stages of development.

Laboratory diagnosis

The laboratory diagnosis 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 to the material 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). It is in these spore balls that the spores (average 2.9 µm x 1.4 µm), the most infective stage of the fungus, are found.¹⁵

In samples where only white 'mummies' have been submitted and spore-producing bodies

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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 (Appendix 5).

Rapid detection and identification of *A apis* can be performed by a polymerase chain reaction using primers specific for *A apis* (Appendix 6).

Varroa mite infestation

The varroa mite, *Varroa destructor*, is a parasite of honeybee colonies in the United Kingdom, Europe, the Middle East, Africa, Asia and the America. It was detected for the first time in New Zealand in April 2000.²² 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.²¹

Pathogenesis

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

Female *V destructor* breed on worker or drone honeybee 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 h later it emerges from the jelly onto the bee, pierces the bee cuticle, and feeds. Approximately 60 h 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, which, 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 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. Consequently, heavy mite infestations result in colony death ('colony collapse').

Epidemiology

The detection of a single female *V destructor* in a geographical region that has previously been declared 'mite-free', indicates that *V destructor* has been present in that region for up to 1 year. Following an initial invasion of *V destructor* into a bee colony, that colony will usually die within 2-4 years if left untreated. Colony death usually occurs when mite infestations become severe, and 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 support extended periods of brood-rearing under poor foraging conditions. Feral honeybee 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 densities. 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.

Clinical Signs

Adult female *V destructor* usually occur inside sealed brood cells or are partly hidden between abdominal segments of adult 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 nurse bees removing infested or dead brood;
- Disfigured and/or stunted adult bees, with deformed wings or legs;
- White coloured debris on the walls or base of capped brood cells;
- Pale or dark reddish spots on otherwise white bee pupae;
- Bees discarding brood from the colony;
- Perforated cell cappings or uncapped cells.

Laboratory Diagnosis

Members of *V destructor* are differentiated from other varroa mites by their behavioural, morphological and genetic characteristics. Of all known varroa mites, only two strains of *V destructor* (the Korea and Japan/Thailand strains) are able to reproduce on *A mellifera*. Other varroa mites, such as *V jacobsoni*, which are native 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. 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.

There are currently 6 recognised strains (or haplotypes) of *V destructor*. These are the Japan/Thailand, Korea, China, Vietnam, Nepal and Sri Lanka strains. Each can be reliably identified from one another only by differences in their mitochondrial DNA (mtDNA) cytochrome oxidase I (CO-I) gene sequences. Methods for obtaining these sequences and confirming the identity of particular strains are described in Appendix 7.

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Appendices

Appendix 1

Microscopic examination for *P l* larvae spores of American foulbrood

- (a) Select two or three diseased individuals that have stretched out on their backs and place them on a clean glass microscope slide. (A match or a small twig can be used to remove the larvae if forceps are not available).
- (b) Pulp the larval remains together on the slide with a pair of forceps.
- (c) Spread the pulped larval remains over the slide.
- (d) Using the side of the forceps push the material off one end, leaving a thin smear of larval material on the slide.
- (e) Air dry the smears; do not leave in direct sunlight.

These air-dried smears can be forwarded to the laboratory without special transport requirements.

When received, process as follows:

- (a) Heat-fix smears.
- (b) Flood the slides with 0.2% carbol fuchsin for 30 s.
- (c) Wash off the stain and allow to air dry or gently blot dry with absorbent material.
- (d) Examine under the microscope for *P l* larvae spores, which are about $1.3 \times 0.6 \mu\text{m}$, ellipsoidal and thick rimmed.

Appendix 2

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

Mix 75 mL of the bulk honey sample with 75 mL phosphate buffered saline (PBS pH 7.2). Smaller volumes of honey can also be used.

Centrifuge the samples at 3000 *g* for 45 min. Pour the supernatant off leaving about 1.5 mL of fluid, which is used to mix with the sediment in the bottles.

Place a 0.5 mL volume in a 5 mL glass bottle and heat at 80°C for 15 min.

Streak a loop full of the sample onto a J agar plate¹⁷ comprising 0.5% tryptone (Oxoid), 0.3% K₂HP0₄ (Ajax), 1.5% yeast extract (Difco), 2% agar (Oxoid), 0.2% glucose (Ajax) and 3 µg/mL nalidixic acid (Sigma). Alternatively sheep blood agar comprising Blood Agar Base No. 2 (Oxoid) supplemented with 7% citrated ovine blood and 3 µg/mL nalidixic acid (SBANa) can be used although SBA will grow only half the number of bacterial colonies that J agar will yield from the same sample¹⁷ (The role of the nalidixic acid is to inhibit the growth of *B alvei* which is commonly found in honey in areas where European foulbrood is endemic).

Colonies appear in 2 to 4 days. After 4 days on J agar colonies are flat, translucent, up to 6 mm in diameter and have an irregular edge. On SBA colonies are flat, grey, about 6 mm in diameter and also have an irregular edge after 4 days.

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Gram stain smears of these colonies show Gram-positive rods, which are 0.5 to 0.6 μ wide x 1.5 to 6.0 μ long. *P l larvae* will sporulate successfully on both J and SBANa plates.

A quick and simple test for the confirmation of the identity of *P l larvae*, once the colony morphology and microscopic criteria have been fulfilled, is a negative catalase test. Catalase positive strains of *P l larvae* are unknown; most other aerobic spore-forming bacteria are catalase positive.¹⁶

Record the number of colony-forming units cultured from the honey samples as '+' if 1 to 20 colonies develop, '++' if 21 to 50 colonies develop and '+++' if >50 colonies develop.

Appendix 3

Preparation of partially purified Sacbrood virus

Large samples (20 or more diseased larvae)

- (a) Grind 20 to 30 diseased larvae in 30 mL of potassium phosphate buffer (PB), pH 7.0, containing 0.02% (0.01 mol/L) sodium diethyldithiocarbamate (DIECA) + 3 mL diethyl ether [(C₂H₅)₂O].¹³
- (b) Emulsify in 3 mL carbon tetrachloride (CCl₄).
- (c) Filter through cotton cheese cloth.
- (d) Centrifuge at 3000 g for 30 min.
- (e) Centrifuge supernatant from (d) at 75 000 g for 2.5 h.
- (f) Resuspend the pellet from (e) in 1 mL PB overnight at 4°C.
- (g) Centrifuge suspended pellet at 8000 g for 10 min.
- (h) The supernatant from (g) can be used in AGID tests and for electron microscopy.

Small samples (five or fewer diseased larvae)

- (a) Extract with 0.1 mL PB/DIECA and 0.1 mL diethyl ether for each larva.
- (b) Centrifuge at 10 000 g for 20 min.
- (c) Let open tubes stand in a fume cabinet for 30 min.
- (d) The supernatant may then be used in AGID tests and for electron microscopy.¹³

Appendix 4

Preparation of antiserum to Sacbrood virus and virus detection by AGID

Antiserum may be prepared in rabbits against virus preparations by giving one intramuscular injection of 0.1 mg of partially purified virus. After 1 week, a second intramuscular injection with about 0.1 mg of partially purified virus in 1 mL of 0.01 mol/L PB at pH 7.0 emulsified with an equal volume of Freund's complete adjuvant is given. A week later a further similar booster injection is given.

Sera that give the highest titres in AGID tests about 6 weeks after the final injection should be stored at –20°C.

AGID tests can then be carried out with the virus extracts and appropriate dilution of sacbrood antiserum using 0.75% agar or agarose in 50 mmol/L PB, pH 6.7, containing 5 mmol/L EDTA and 0.2% sodium azide (NaN₃).

Appendix 5

Culture of *Ascospaera apis* for diagnosis of Chalkbrood

Yeast-glucose-phosphate agar is comprised of 1% yeast extract (Oxoid), 0.1% cysteine or cystine (BDH), 1% glucose (Ajax), 1.35% KH₂P₀₄ (Ajax) and 1% soluble starch (Ajax).¹³ The plates should be incubated at 37°C in an atmosphere containing 5 – 10% CO₂. Fungal colonies grow moderately slowly and are 5 – 7 cm in diameter after 10 days. They produce aerial mycelia deeply floccose or matted, white to pale buff and may become coral to pale reddish brown with age.¹⁸

To confirm the identity as *A apis* it is necessary to mate it with one of two known mating strains of *A apis*.¹⁹ 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 –10 days, however, the production of spore balls and the spores within make take up to 15 days.

Appendix 6

PCR for the detection and identification of *Ascospaera apis*

DNA is extracted from the suspect fungus by either a rapid or slow DNA extraction method. For the rapid method, suspect 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 shaken vigorously for 30 s and centrifuged at 6000 g for 2 min in an Eppendorf bench centrifuge. The supernatant is then removed to a clean PCR tube and used directly in the PCR reaction.

For the slow extraction method, which produces cleaner DNA preparations than the rapid method, 0.4 to 0.5 g of compressed fungus mycelia are ground in liquid nitrogen to a fine powder in a pre-cooled mortar and pestle. 3 mL of freshly prepared K buffer (2.0 mg/mL proteinase K, 0.1 M Tris-HCl 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°C for 1 h, 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 min.

The phases are then separated by centrifugation at 2000 g for 10 min, 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 4°C at 6000 g for 10 – 15 min, resuspended in 75% ethanol, pelleted again at 6000 g for 5 – 10 min, dried at room temperature, resuspended in 500 µL TE containing 10 pg/mL RNase A and incubated at 37°C for 30 min. 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. Precipitated DNA is pelleted, resuspended and dried as described above, resuspended in 200 µL of distilled H₂O and used directly in the PCR reaction or frozen at -20°C until needed.

The nuclear rDNA region containing 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'-GCTAGGTGCCCTAAACAAGGC- Y(CBP1) or 5'-TTTGAGTTCCCCCTGGCTAGC- 3' (CBP2) in conjunction with the reverse primer 5'-ACTAGAGCGAAAGACAAAGCC- 3' (CBP3) using methods

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described by Anderson et al.²⁰ These primers were constructed from original sequence data generated by Anderson et al.²⁰ 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 to 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 1 drop of oil. DNA is amplified using an automated thermal cycler (Corbett Research, Model FTS-1) and the following protocol: 5 min initial denaturation at 94°C, after which 2 U of Taq polymerase is added, then 30 cycles of 1 min at 94°C, 1.5 min annealing at 54 to 55°C and 2 min extension at 72°C. A final extension period of 5 min at 72°C completes the amplification. Five microlitres 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 about 3 h. DNA bands are then visualised using an ultra-violet transilluminator.

Appendix 7

Detection and identification of *Varroa* mites by mtDNA sequencing

All known genotypes of *Varroa* can only be reliably identified from their particular mtDNA CO-I gene sequences.

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 pH8.3, 5 mM MgCl₂, 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°C for 30 min then at 95-100°C for 10 min, diluted with 40 µL dH₂O and 2-20 µL used as template in 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)AATAGCAAAT

AC- 3' (COXRa), and following methods described in Appendix 6.

Amplified DNA is then sequenced directly 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 them 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 for the common Korea and less common Japan/Thailand strain of *V destructor* are AF106899 and AF106897, respectively.