

# DIAGNOSIS AND DETECTION OF DIFFERENT GENOTYPES OF *PAENIBACILLUS* LARVAE, THE CAUSAL AGENT OF AMERICAN FOULBROOD DISEASE

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American foulbrood (AFB) is the most contagious and destructive infectious disease affecting the larval and pupal stages of honey bees (*Apis mellifera*) and other *Apis* species. The causative agent, *Paenibacillus larvae*, is a Gram-positive bacterium that can produce over one billion spores in each infected larva. AFB occurs in temperate or subtropical regions throughout the world and leads to huge losses not only in the apicultural economy but also in pollination rates since *Apis mellifera* is the most widely used actively managed pollinator in the world. The disease is classified within the World Organization for Animal Health (OIE) list and considered to be of socio-economic impact and significance in the international trade of bees and bee products.

Only bacterial spores are capable of inducing the infection. Spores can remain viable for long periods and survive adverse conditions (desiccation, high temperatures, ultraviolet light exposure) and contact with standard disinfectants. The spread of the disease is facilitated by normal beekeeping practices, including exchanging hive components between colonies, maintaining many hives in a confined area, and trading queens, package bees and honey.

## Clinical signs

Clinical signs are diverse and depend on the genotype involved. In severely infected colonies, the combs have a mottled appearance caused by a pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. The capping of a cell that contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses. Also, the larva or pupa changes their color, first to beige and eventually to a dark brown. The larvae can become glutinous in consistency and can be drawn out as threads when a probe is inserted into the larval remains and removed from the cell (match-stick test). Ropy test is probably the best-known technique for field diagnosis of the disease, but in some cases the larval remains are rather watery, resulting in a negative match-stick test. Finally, one month or more after the larva becomes ropy, the remains of the diseased brood dry out to form typical hard, dark scales that are brittle and adhere firmly to the lower sides of the cell. If death occurs in the pupal stage, the pupal tongue protrudes from the pupal head, extending to the top of the brood cell or may angle back towards the bottom of the cell. The protruding tongue is one of the most characteristic signs of the disease, although it is rarely seen. The tongue may also persist on the dried scale.

## Diagnosis

Traditional methods such as the recognition of typical clinical symptoms of infection, culture of *P. larvae* from diseased brood, and microscopy provide efficient and inexpensive means of diagnosing the disease. Clinical diagnosis of AFB is based on the identification of the pathogenic agent by microscopic examination of stained smears of dead or sick larvae with 0.2% carbol fuchsin. Ellipsoidal spores 1.3 X 0.6 µm in average are observed after staining. Symptoms of infection are easily recognizable in freshly dead larvae from diseased colonies.

A commercially available lateral flow device for the detection of AFB using monoclonal antibodies has been developed and can identify infected larvae in the field within 10 minutes without the need for special equipment, but it has not yet been validated.

Apart from the distinctive clinical symptoms, laboratory confirmation of the presence of *P. larvae* is required in most countries where AFB is a notifiable disease. For laboratory diagnosis, spores can be isolated and cultured from various sources including ropy larval remains, scales, honey, pollen, wax and adult bees. Routine analysis of honey samples or adult bees for viable spores of the pathogen is strongly recommended to detect not only subclinical infections but also different *Paenibacillus larvae* genotypes that do not exhibit typical clinical signs.

### Isolation and culture

#### *Diseased larvae*

In general, an aqueous solution containing *P. larvae* spores should be prepared for further analysis. This spore suspension is heat-shocked at 80°C for 10 minutes or 95–96°C for 3–5 minutes to kill vegetative forms of other microorganisms, including other spore formers. Different genotypes of *P. larvae* show variation in germination ability, and their response to heat treatment is variable (Forsgren *et al.*, *Vet. Microbiol.*, 2008). The germination rate of endospores of genotype ERIC I is strongly stimulated by heating to 90–95 C for 10 min while ERIC II genotype responds negatively to heat treatment (also 80–85–90–95 C). Also, ERIC II genotype spores lose viability after 6 months storage, a clear difference with ERIC I genotype strains.

Larval/pupal remains from brood comb are collected with a sterile swab and suspended in 5–10 ml of sterile water or physiological solution in a test tube. For larval or pupal remains submitted on a glass slide, add 2–3 drops of sterile water. Emulsify with a wooden stick sterile loop. Place a loopful of emulsified material onto a suitable agar plate and streak with a sterile loop to obtain isolated colonies. Plates are incubated in 5–7% CO<sub>2</sub> and examined daily for up to seven days. Colonies are visible from day 2 onwards.

Each spore-suspension must be divided in triplicate:

- a) without heat treatment;
- b) with heat treatment at 80°C for 10 minutes;
- c) with heat treatment at 95°C for 3 minutes.

The heating step is for killing vegetative forms of other microorganisms. This significantly reduces the risk that *P. larvae* colonies will become masked by these competitors. Nevertheless, bacteria of the genera *Bacillus*, *Paenibacillus* and *Brevibacillus* may continue to swarm over the plates which necessitate the use of semi-selective media through the addition of the antibiotics nalidixic acid and pipemidic acid In step a) (no heat treatment), as well as both antibiotics, amphotericin B or cycloheximide should be used to avoid fungal contamination in the isolation plates. Incubate the plates at 36±1°C for 2–4-days.

For smears prepared from dead larvae, add 1–2 drops of sterile water and mix on the slide. Use a wire loop to prepare a new smear for Gram stain and microscopic examination for spores. A second loop of reconstituted material is used to culture onto a suitable agar plate with the addition of antibiotics and cycloheximide

*Honey:* Honey samples are heated to 45–50°C and shaken to distribute any spores that may be present, then each honey sample should be diluted (1/1) in PBS (0.01 M pH 7.2) or physiological saline, transferred to a centrifuge tube and centrifuged at 6000 *g* for 40 minutes. The supernatant is discarded leaving approximately 3 ml per tube and vortex-mixed for 1 minute to re-suspend the pellet. The suspension must be treated as described for larval remains. The samples are vortex-mixed again for 2 minutes, and 100 µl of the sediment-fluid mixture poured on suitable culture media with

the addition of antibiotics (and cycloheximide for non-heated samples) and incubated at 37°C for 7–8 days.

**Media:** Several media for cultivating *P. larvae* have been described: PLA (*Paenibacillus larvae* agar); MYPGP (Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, and pyruvate) agar; BHIT agar (brain–heart infusion medium supplemented with thiamine); J-agar and CSA (Columbia sheep-blood agar). Antibiotics can be added to any of the media.

On PLA, colonies of *P. larvae* are small, pale green to yellow (= the same color as the medium), with a slightly opaque and rough surface; sometimes the center is raised. On MYPGP and J-agar, colonies are small, regular, mostly rough, flat or raised and whitish to beige colored. On CSA agar, colonies are small, regular, rough, butyrous and grayish, somewhat transparent and slightly glistening appearance.

*Paenibacillus larvae* colonies with orange to red pigmentation have been described, i.e., ERIC II and III genotypes that produce reddish and/or orange colonies on MYPGP, J-agar, and CS. According to Genersch *et al.* (2005; 2006) only genotypes ERIC II and III are pigmented, but pigmented and non-pigmented strains of ERIC IV has been reported (Dingman, 2015). It is important to point out that pigmented phenotypes can be lost under successive sub-culturing.

### Molecular methods

In the last 25 years, considerable progress has been made in the understanding and taxonomic reclassification of the causative agent, as well as in the diagnosis of AFB. Diverse genotypes of the pathogen with different virulence have been identified using rep-PCR fingerprinting and exposure bioassays to infect young larvae, as well as the previously named *Paenibacillus larvae* subsp. *pulvifaciens* (syn. *Bacillus pulvifaciens*) strains, which cause 'powdery scale disease.'

Several PCR methods, including real-time PCR, for identification and genotyping of the pathogen from brood and bee products, have now been extensively developed.

Specific PCR may be used to identify the bacterial colonies isolated in culture media or for rapid confirmation of clinical signs of AFB (larval remains, scales).

**DNA from bacterial colonies:** We use a rapid procedure by using whole cells from plates. Suspend cells from 1-2 single colonies in 300 µl double-distilled sterile water, then vortex mixed and centrifuged at 14,000 rpm for 4 min, the supernatant is removed, and the pellet resuspended in 150 µl of an aqueous suspension of 6% resin Chelex 100 (Bio-Rad). The mixture of cells and resin is incubated at 56°C for 20 min, vortex mixed, incubated at 99°C for 15 min, and vortex mixed for 1 min. Finally, bacterial debris and resin are removed by centrifugation and the supernatant used as template for PCR.

**Larval samples:** 1-2 diseased larvae in 1 ml sterile distilled water emulsified and mix well. Dilute 100 µl with 900 µl distilled water, vortex mix, and use to extract DNA by heating and centrifugation. It is possible to use an instant gene matrix (Chelex)

**Honey:** Heat 5 ml of honey at 40°C, mix with an equal volume of PBS, centrifuge at maximum speed for 20 min, discard the supernatant, resuspend pellet in buffer (DNAeasy Plant mini kit Qiagen) and follow the protocol of manufacturer's. Use the DNA as a template for PCR

**rep-PCR:** The availability of standardized techniques that allow the discrimination of different *P. larvae* strains is essential for studying the epidemiology of AFB. This will allow scientists to identify outbreaks of the disease, determine the source of infection, determine the relationship between outbreaks, recognize more virulent strains, and monitor prevention and treatment strategies. PCR

amplification of repetitive elements present in bacterial DNA (rep-PCR) is useful for genotyping. There are three sets of repetitive elements randomly dispersed in the genome of bacteria, enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) elements, and BOX elements (which includes boxA, boxB, and boxC). Primers to amplify those elements have been reported and proved to be useful for subtyping of Gram-positive and Gram-negative bacteria. ERIC-PCR amplification has shown four *P. larvae* genotypes, named ERIC I, II, III, and IV. This typing scheme correlates with phenotypic differences including spore surface configuration, colony morphology, and virulence. Genotypes ERIC I and II are regularly isolated from infected colonies worldwide, whereas ERIC III and IV are only represented by few isolates in Type Culture Collections. Only ERIC I and II are of practical importance.

Excluding brood symptoms, comparison of phenotypic characteristics link ERIC II more closely to ERIC III and IV (*Plp*) and clearly separate it from ERIC I (*PII*). Pulse field electrophoresis profiles and SDS-PAGE of whole cell proteins also distinctly linked ERIC II with ERICs III and IV. *P. larvae* ERIC II genotype is best grouped with ERICs III and IV as being associated with *Plp* strain. In my opinion, ERIC I comprises "classical" *PI* strains, and the other 3 ERIC groups include the former *Plp*.

Also, by using a multilocus sequence typing (MLST) scheme based on 7 loci, Morrissey and co-workers (2014) separate *P. larvae* isolates in 23 sequence types that grouped into 3 clusters, again segregated ERIC I (classical *PI*) from the other 3 ERIC genotypes. In addition, *P. larvae* isolates classed as ERIC I were more diverse than those classed as ERIC II, containing 16 unique STs compared with only 3 for ERIC II. ERIC II (a third subspecies?) formed a cluster independent of ERIC I with ERICs III and IV (formerly *P. pulvifaciens*) forming a third cluster. Using a similar approach, Krongdang et al. (2017) amplified 7 housekeeping gene loci and obtained 15 sequence types of *P. larvae* isolates within a collection of 38 isolates (from North America & reference strains from collections). The data presented using the MLST scheme strongly supports this technique as a powerful novel method for studying *P. larvae* population genetics and epidemiology.

Comparison of genomes between an ERIC I isolate and an ERIC II isolate has shown 13% more DNA (approximately 1000 genes) in the ERIC I (Djukic et al., 2014).

#### Disease Cycle

Spores transmit the disease and are infectious only for larvae, not for adult bees. Very young larvae (12-36 hours after hatching) are the most susceptible. During this time window, the oral uptake of 10 spores or fewer is sufficient to cause disease, but susceptibility rapidly decreases when the larvae get older. Larvae become infected by ingesting bacterial spores - either as a result of larvae being reared in cells that previously contained infected larvae or as a result of larvae being fed by food contaminated with spores that have been picked up by cell-cleaning bees that later assume nurse bee duties. Ingested spores germinate in the larval midgut lumen. Vegetative bacteria massively proliferate within the midgut lumen living like commensals from the food ingested by larvae (2 to 6 days after ingestion). During this non-invasive phase, no damage of the epithelial cells is observed although the peritrophic membrane matrix (PM) may be affected. During this stage of infection, *P. larvae* follows a commensal lifestyle living from the food ingested by the larvae. The pathogen contains enzymes involved in carbohydrate metabolism and can metabolize different sugars including glucose and fructose to support vegetative growth. At this point, the larval gut contains nothing but these pathogenic bacteria. The invasive phase of the infection starts when the peritrophic matrix is degraded, and the bacteria breach the epithelium and invade the hemocoel causing the larval death. This step coincides with larval death. *P. larvae* use the paracellular route for crossing the midgut epithelium and enter into the organ cavity. *P. larvae* eliminate bacterial competitors in the larval gut by releasing non-ribosomal peptides. Subsequently, the peritrophic membrane [that contain chitin] (PM) that protects the underlying gut epithelium is attacked by chitin-degrading enzymes, one of which was recognized as a critical virulence factor. These

characteristics are shared by both ERIC I and II genotypes. Total degradation of the peritrophic matrix is the key step during pathogenesis.

ERIC I strains expressed two toxins that attack the gut epithelium. These toxins are virulence factors that act on the epithelial cells once the protective peritrophic matrix has been degraded. Bacteria then breach the epithelial cells via the paracellular route and invade the hemocoel. ERIC II genotype express an S-layer protein that mediates adhesion of the bacteria to the midgut epithelial cells. The steps following adhesion remain unclear.

Irrespective of the strategy, *P. larvae* infection with both genotypes result in larval death. After the infected larvae have died from AFB, proteases released by the bacteria degrade the larval remains to a brownish, semi-fluid glue-like colloid, adheres to an object, such a toothpick and strings out in a thread of gummy substance for a considerable distance (*ropy stage*) and giving the colony a “foul” look and smell. Sporulation takes place in this environment. As time passes, single not-removed larva or pupa dries down to a hard scale, which adheres firmly to the bottom of the cells and is very difficult for the bees to remove. One single scale contains about two and a half billion spores.

Different genotypes differ in virulence; comparative exposure bioassays demonstrated that ERIC II is more virulent on the larval level than ERIC I.

ERIC I strains need up to 12 days to kill all infected larvae, while ERIC II, III and IV strains kill infected larvae within 6- 7 days. The faster the pathogen kills infected larvae; the more infected larvae will be removed as nurse bees seem to recognize dead larvae at lower rates after cells capping. The proportion of larvae that develop into a ropy mass under the cell cappings is higher for infections of genotype ERIC I. At colony level; nurse bees removed ERIC II- infected larvae more efficiently than the slower ERIC I-infected larvae, so ERIC II strains are less virulent on the colony level than ERIC I. As veterinarians and beekeepers look for a ropy mass inside capped cells as the main sign of the disease, thus false negative diagnoses are likely to occur if colonies are infected with strains of genotype ERIC II because only a few infected cells may be present and a more thorough inspection of the honey bee brood nest is required

*Explanation of Figure: A Proposed molecular model for the pathogenesis of P. larvae infections: 1) During colonization of the midgut lumen, secondary metabolites secreted by P. larvae [patterned hexagons] i.e., non-ribosomal peptide antibiotics; (e.g., paenilamicin and iturin) eliminate bacterial and fungal competitors in order to make P. larvae the only bacterium present, and set the stage for conquering the larval midgut lumen. 2) The first step in attacking the midgut epithelium is the degradation of the peritrophic matrix through the chitin-degrading enzyme PICBP49 (red stars) to digest the peritrophic matrix (PM, pink structure) which should protect the midgut epithelium (gray cells with blue nuclei) against pathogen attack. Once the midgut epithelial cells are no longer protected by the PM, P. larvae can attack the epithelium. This step might also serve nutritional purposes and marks the transition from the non-invasive to the invasive phase of infection. 3.1. P. larvae ERIC I attacks the unprotected epithelial cells via secreted toxins (purple ovals) which interfere with cellular functions thereby destroying the epithelial integrity and enabling bacteria to breach the epithelium via the paracellular route. 3.2. P. larvae ERIC II expresses an S-layer protein SplA (yellow edge) on its surface mediating direct attachment of P. larvae ERIC II to epithelial cells. This step seems to be important for the bacteria to breach the epithelium using the paracellular route via a/ non-identified mechanism/s. Finally, invasion of the hemocoel coincides with larval death. P. larvae continues to proliferate thereby completely decomposing the larval cadaver. The siderophore bacillibactin might play a role during this phase, and peptide antibiotics might prevent saprophytes to take over.*

AFB Infection is spread by two main routes: first, 8-19% of larvae reared in cells, which previously contain infected larvae become infected themselves, and second, cell-cleaner bees transmit spores

to larval food when they became nurse bees. Bacteria proliferate in the larval tissues before pupation, and infected larvae quickly die and spore form, mostly in prepupae 11-12 days-old after egg hatching for ERIC I and in larvae of 6-7 days old for ERIC II. The transfer of AFB from one colony to another is an essential step in the disease cycle. Transmission of AFB is possible both vertically and horizontally in some ways. AFB can be horizontally transmitted by letting bees robbed contaminated colonies, feeding infected honey and pollen; transferring frames of brood from diseased colonies to healthy ones; using hive equipment with at some time has been contaminated with *P. larvae* spores; installing infected package bees or queens and, in a minor way by drifting bees. Also, vertical transmission of spores between colonies is possible through swarming.