AMERICAN FOULBROOD OF HONEY BEES (INFECTION OF HONEY BEES WITH *PAENIBACILLUS LARVAE*)

SUMMARY

Description of the disease: *American foulbrood (AFB) affects the larval stage of the honey bee* Apis mellifera *and other* Apis *spp., and occurs throughout the world.* Paenibacillus larvae, *the causative organism, is a bacterium that can produce over one billion spores in each infected larva. The spores are extremely resistant to heat and chemical agents, and can survive for many years in scales (from diseased dead brood), hive products and equipment. Only the spores are capable of inducing the infection.*

Combs of infected colonies have a mottled appearance due to a mixture of healthy capped brood, unhealthy capped cells and uncapped cells. This is not a characteristic of AFB only. Cell cappings of a diseased larva appear moist and darkened, becoming concave and possibly punctured as infection progresses. The larval or pupal colour changes to creamy brown and then to a dark brown with a ropy appearance when drawn out. In some cases the larval remains are rather watery. The diseased brood eventually dries out to form characteristic brittle scales that adhere tightly to the lower sides of the cell. The formation of a pupal tongue is one of the most characteristic but rarely seen signs of the disease and precedes the formation of the scales. The clinical signs of AFB are very diverse and depend on the genotype involved, the stage of the disease and the strength of the bee colony (and possibly its resistance to AFB). Genotypes ERIC I–V are all pathogenic for honey bees

Detection and identification of the agent: *Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs. The analyst can make use of a broad range of sample types. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. Some of the identification methods require a previous culturing step, while others can be performed directly on collected samples. Five solid culture media are recommended: PLA (Paenibacillus larvae agar), MYPGP agar, BHIT agar, J-agar and Columbia sheep blood agar. Two polymerase chain reaction (PCR) protocols are described in this chapter that can be used for rapid confirmation of clinical AFB and for identification of bacterial colonies after a cultivation step. The biochemical profiling of P. larvae is based on the catalase test, the production of acid from carbohydrates and the hydrolysis of casein depending on the genotype involved. Further, antibody-based techniques and the microscopic identification of the pathogenic agent are described.*

Serological tests: *There are no serological tests available.*

Requirements for vaccines: *No vaccines are available.*

A. INTRODUCTION

American foulbrood (AFB) is an infectious disease of the larval stage of the honey bee *Apis mellifera* and other *Apis* spp., and occurs throughout the world where such bees are kept. *Paenibacillus larvae*, the causative organism, is a Gram-positive bacterium that can produce over one billion spores in each infected larva. The bacterium is a round-ended, straight or sometimes curved rod, which varies greatly in size (0.5–0.8 μm wide by 1.5 –6 μm long), occurring singly and in chains and filaments; most strains are motile. The sporangia are often sparse in vitro, and the

ellipsoidal, central to subterminal spores, which may swell the sporangia, are often found free, measuring 0.6 × 1.3 μm (Heyndrickx *et al.*, 1996).

By using repetitive element polymerase chain reaction (rep-PCR) and primers ERIC1R-ERIC2, five different genotypes (ERIC I, II, III, IV and V) are differentiated (Beims *et al.*, 2020). Genotypes ERIC I and II correspond to the former subspecies *P. l. larvae* while genotypes ERIC III and IV correspond to the former subspecies *P. l. pulvifaciens* (Genersch, 2010). Genotype ERIC V was recently isolated from a Spanish honey sample and identified as a new genotype (Beims *et al.*, 2020). All five genotypes differ in colony and spore morphology, in their metabolism of carbon sources and most importantly in virulence. Exposure bioassays revealed that members of ERIC II-V are highly virulent against larvae in terms of the time course of mortality. Almost all larvae infected with these genotypes are killed within approximately 3–7 days (Beims *et al.*, 2020; Genersch *et al.*, 2005; Rauch *et al.*, 2009). This means that only a minor proportion of the larvae die after cell capping resulting in the described clinical signs of AFB (ropy stage, foulbrood scale). In contrast, genotype ERIC I usually takes around 12 days to kill an infected larva and is therefore considered less virulent than ERIC II-V for the individual larva (Beims *et al.*, 2020; Djukic *et al.*, 2014; Genersch, 2010; Genersch *et al.*, 2005). Epidemiological studies showed that only ERIC I and ERIC II are frequently isolated from AFB-disease colonies. *Paenibacillus larvae* genotype ERIC I is the most frequent genotype while genotype ERIC II seems to be more restricted although both have been reported worldwide. Genotypes ERIC III and IV have not been identified in the field for decades, but exist as a few isolates in culture collections (de Graaf *et al.*, 2013; Genersch, 2010). Genotype, ERIC V was recently isolated from a Spanish multi-flower honey sample. Because fingerprinting profiles generated via electrophoresis of rep-PCR amplified DNA are not consistently reproducible between laboratories, it is necessary to include reference *P. larvae* strains previously typed. To enhance the discrimination of strains, the analysis using ERIC primers can be complemented with the use of other primers (de Graaf *et al.*, 2013). A multilocus sequence typing (MLST) scheme revealed the distribution and biogeography of 294 samples of *P. larvae,* grouped in 34 sequence types (as of March 2022 [https://pubmlst.org/plarvae/]), across six continents (Morrissey *et al.*, 2015). Currently, whole genome sequencing (WGS) based genotyping offers unprecedented resolution in discriminating highly related strains. Thus, it is well suited for the investigation of the evolution of pathogens even over short time periods (Uelze *et al.*, 2020). Applied to the investigation of *P. larvae*, WGS-based genotyping showed a good correlation with conventional typing methods (MLST and ERIC-PCR) and proved to be efficiently used for cluster delineation in AFB outbreak epidemiological investigations (Bertolotti *et al.*, 2021). The spores are extremely heat stable and resistant to chemical agents. Only spores are capable of inducing the infection. The infection can be transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell. Although the larval stages of worker bees, drones and queens are susceptible to infection, infected queens and drone larvae are rarely seen under natural conditions. The susceptibility of larvae to AFB disease decreases with increasing age (de Graaf *et al.*, 2013); larvae cannot be infected later than 53 hours after the egg has hatched. The mean infective dose (LD$_{50}$= spore dose at which 50% of the larvae are killed) needed to initiate infection, though very variable, is 8.49 ± 1.49 spores in 24–28 hour-old bee larvae (Hansen & Brodsgaard, 1999). Exchanging combs containing the remains of diseased brood is the most common way of spreading the disease from colony to colony. In addition, feeding or robbing of spore-laden honey or bee bread, package bees and the introduction of queens from infected colonies can also spread the disease. Wax contaminated with the spores of *P. larvae*, used in the production of comb foundations, can also spread the disease if not properly treated. The early detection of AFB helps to prevent further spread.

While there is generally a low risk of human infection with AFB organisms, it should be noted that fatal bacterial septicaemia has been reported in drug users injected with honey contaminated with *P. larvae* spores (Rieg *et al.,* 2010). Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

1. Epizootology and clinical signs

Spores of *P. larvae* can survive in bee products (honey, wax, dead larvae) and in the environment for 3 to 10 years and for 35 years in dry larval scales. Purified spores can survive even more than 70 years.

The clinical signs of AFB are very diverse and depend on the genotype involved, the stage of the disease and the strength of the bee colony (and possibly its resistance to AFB) (Genersch *et al.*, 2005). Larvae can be killed rapidly at an early age when they are curled at the base of uncapped brood cells. Adult worker bees will remove these dead larvae leaving only an empty cell (Brodsgaard *et al.*, 2000). Other larvae will die later on in their development, when they are in an upright position, filling most of the brood cell. Larvae infected with ERIC I usually die after brood cell capping, whereas larvae infected with other types usually die before cell caping.

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 (Figure 1a). Also, the larva or pupa changes colour, 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) (Figure 1b). This 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, 1 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 strongly to the lower sides of the cell (Figures 1.d. and 2). 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 (Figure 1c). The tongue may persist also on the dried scale. European foulbrood needs to be taken into consideration as a differential diagnosis.
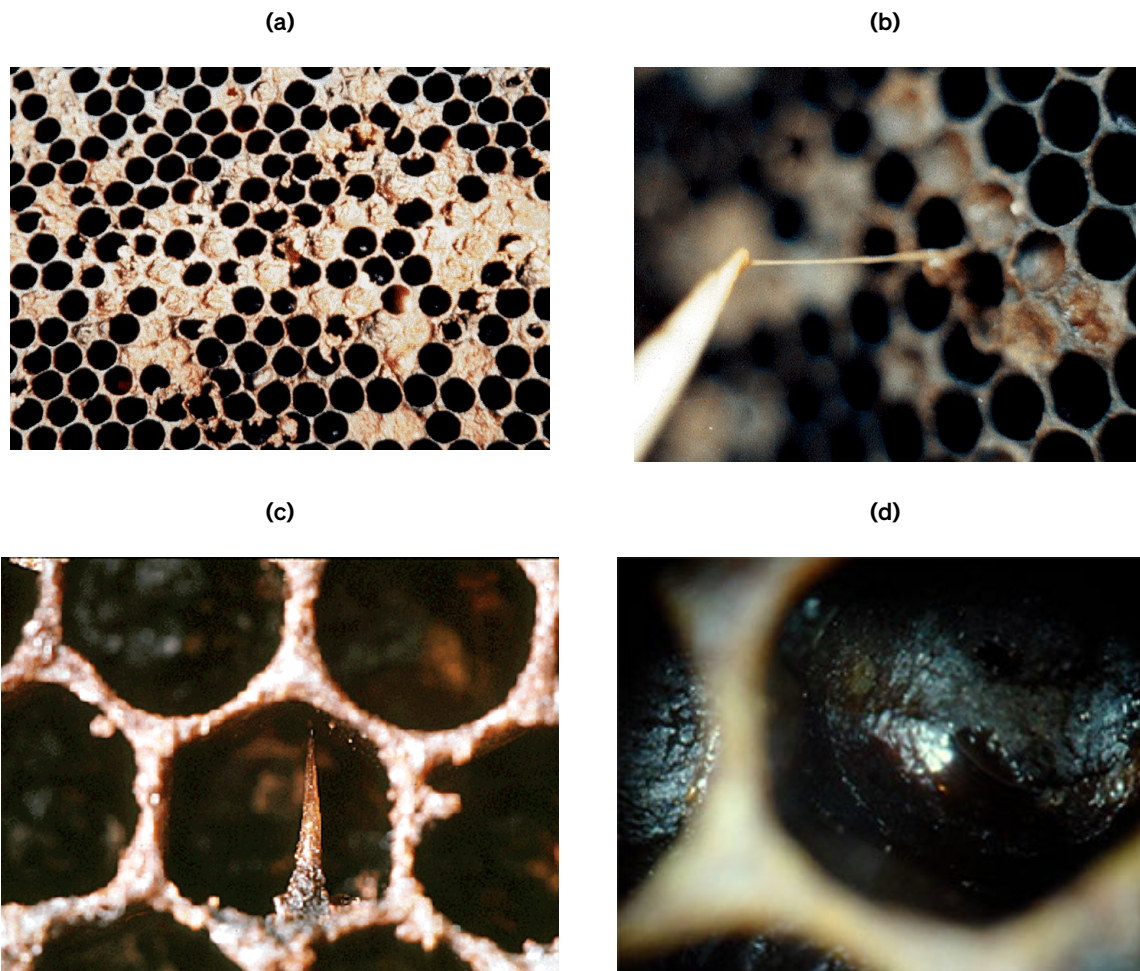
(a)

(b)

(c)

(d)

Fig. 1. Clinical American foulbrood (*a–d*): (*a*) *Combs have mottled appearance.* (*b*) *A matchstick draws out the brown, semi-fluid larval remains in a ropy thread.* (*c*) *The formation of a pupal tongue is a very characteristic sign, but rarely seen.* (*d*) *Residual scale adhered to the bottom of the cell. Photos a, b and d From: A. M. Alippi; Photo* (*c*): *From: MAAREC-Mid Atlantic Apiculture and Extension Consortium* (*at* https://agdev.anr.udel.edu/maarec/honey-bee-biology/honey-bee-parasites-pests-predators-and-diseases-of-honey-bees/nggallery/show--photocrati-nextgen_basic_thumbnails/page/1/)
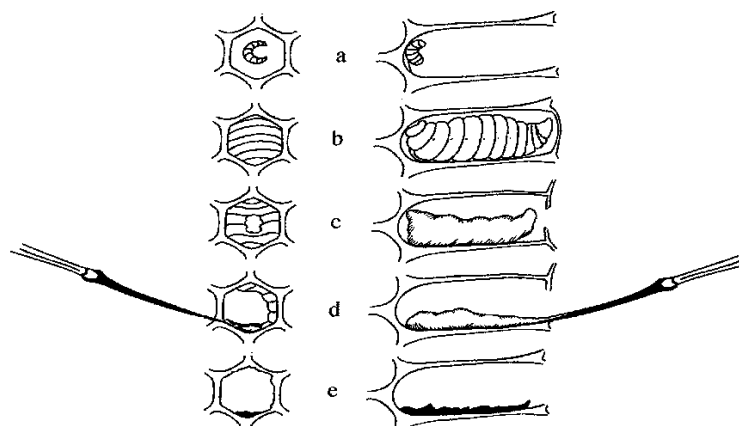
*Fig. 2. Progression of the disease: (*a*) Point of infection. (*b*) Larval development to the prepupal stage.*
*(*c*) Cell contents reduced and capping is drawn inwards or is punctured. (*d*) Cell contents become glutinous.*
*(*e*) Residual scale tightly adherent to bottom of cell.*

It has been demonstrated that different genotypes differ in virulence; ERIC I strains lead to 100% mortality of infected larvae within 12 days while ERIC II strains kill infected larvae in about 7 days (Djukic *et al*., 2014; Genersch, 2010; Genersch *et al*., 2005; Rauch *et al.,* 2009). The faster *P. larvae* kills infected larvae, the more infected larvae will be removed as nurse bees seem to recognise dead larvae at lower rates after cells capping (Rauch *et al.*, 2009). Therefore, the proportion of larvae developing into a ropy mass under the cell cappings is higher for infections with strains of genotype ERIC I but can still be seen with other ERIC types. As veterinarians and beekeepers look for a ropy mass inside capped cells as the main sign of the disease, false negative diagnoses are likely to occur if AFB-diseased colonies are infected with strains of genotype ERIC II because only a few infected cells may be present (Rauch *et al.*, 2009).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of American foulbrood and their purpose

Method	Purpose					
	Population freedom from infection	Individual hive freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[a]						
Bacterial isolation	+++	+++	+++	+++	+++	–
Microscopy	++	++	++	+++	+++	–
Antigen detection	++	++	++	++	++	–
Conventional PCR	+++	+++	+++	+++	+++	–
Real-time PCR	+++	+++	+++	+++	+++	–
Mass spectrometry	–	–	–	++	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.

1.   Detection and identification of the agent

Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs. The analyst can rely on a broad range of samples. However, in practice, the samples of choice will depend on whether it concerns a suspicious or diseased honey bee colony/apiary, or analysis in the context of an AFB monitoring/prevention programme. An initial overview of clinical signs of the disease is provided in section A of this chapter. Identification techniques are microbiological characterisation, the polymerase chain reaction (PCR), biochemical profiling, antibody-based techniques and microscopy. The analyst should be aware of differences in sensitivity between the presented approaches and should select the most appropriate for a given situation.

1.1.   Selection of samples

1.1.1.   Collection of samples from a suspicious or diseased colony or apiary

While maintaining their colonies, beekeepers often find brood combs with signs of disease. In this case a brood sample can be collected for diagnosis. If possible, submit a whole frame to the laboratory, to avoid risk of distortion in transit. Alternatively the brood may be sampled by cutting out a piece comb of at least 20 cm^2 in size, containing as much of the dead or discoloured brood as possible. An experienced person can collect infected larval or pupal remains directly from the cells with a sterile swab, significantly reducing the sample size and facilitating packaging and sample transportation to the laboratory (see below). When microscopic examination is the method of choice, smears of the remains of diseased larvae can also be made at the apiary (de Graaf *et al.,* 2013). After air-drying the smears are packaged to be forwarded to the laboratory for microscopic examination and culture.

Every bee colony in the vicinity of such a clinical case of AFB should be considered as suspicious and a broad range of samples should be taken for confirmation. Apart from brood samples, food stores (honey, pollen and royal jelly), adult workers and wax debris can be used to detect the presence of *P. larvae* spores. Honey samples can be collected from cells close to the brood with separate disposable spoons to prevent cross-contamination between samples; however, honey may have been sitting in the comb for months at the time of sampling. Adult bees can be shaken or brushed from the combs of the brood chamber or the honey supers into a plastic bag or container. For the most reliable picture of the actual situation, bees from the brood nest (and not the honey supers) should be analysed. Wax debris can be collected at the hive bottom all year round.

1.1.2.   Samples for AFB monitoring or prevention programmes

To prevent the propagation of diseased brood, honey, adult bee and debris samples can be used to detect AFB in colonies where no clinical signs are observed. Routine collection of samples from colonies or from harvested honey can be used as part of an operational or regional AFB detection programme.

Microscopic examination of smears from larvae with no clinical signs is far less sensitive at detecting spores in colonies compared with bacteriological or PCR-based methods. In fact, bacteriological and PCR-based methods will often detect spores in colonies that never develop clinical signs of AFB. High numbers of spores cultured from honey and bee samples using bacteriological methods, however, can often predict the presence of clinical AFB signs at colony, apiary and operational levels.

1.2.   Packaging and transportation of samples to the laboratory

Brood comb should be wrapped in a paper bag, paper towel or newspaper and placed in a wooden or heavy cardboard box for transport; avoid any form of plastic wrapping to prevent fungal growth. Swabs with larval remains can be put into appropriate test tubes with a cap. Smears of dead larvae on microscope slides are placed into individual slide holders. These are commercially available. Adult bees can be kept frozen or submerged in 70% ethanol in leak-proof containers during transportation, although dried bees are adequate, a sample size should be at least 30 bees. Food supplies can be put into a test tube or a suitable pot, or wrapped in a plastic bag together with the spoon. Leaking and cross-

contamination of the samples must be prevented. If possible, fresh material for laboratory tests should be sent refrigerated.

Hive debris and wax should be wrapped in a paper bag, plastic pots covered with a paper lid or in paper tubes covered with a plastic lid. Secondary packaging consists of a plastic bag as a protection against cross-contamination. In the case of bulk samples, samples may be stored even in tertiary packaging (large cardboard boxes) that protects samples from mechanical damage.

The minimum amount of honey for detection of viable spores of *P. larvae* is 50 g and should be placed in a leak-proof plastic container (one per sample with an identification label).

1.3. Culture

1.3.1. Sample preparation

i) Samples for cultivation

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 in order 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.,* 2008). Direct plating of larval remains for cultivation on agar without heat treatment is possible.

Larval/pupal remains from brood comb are collected with a sterile swab and suspended in 2–10 ml of sterile water or physiological solution (0.01 M phosphate-buffered saline [PBS] or 0.9% NaCl) in a test tube. For larval or pupal remains submitted on a glass slide, add 2–3 drops of sterile water. Emulsify with an orange stick or 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_2 and examined daily for up to seven days. Colonies are visible from day 2 onwards.

Each spore-suspension sample should be divided and treated as follows:

a) without heat treatment;

b) with heat treatment at 80°C for 10 minutes;

and

c) with heat treatment at 95°C for 3 minutes.

Steps b) and c) are for killing vegetative forms of other microorganisms. The heat step will significantly reduce 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 necessitates the use of semi-selective media through addition of the antibiotics nalidixic acid and pipemidic acid. In step a) (no heat treatment), as well as both antibiotics, amphotericin B at a final concentration of 16.8 µg/ml of culture medium should be used to avoid fungal contamination in the isolation plates. Incubate the plates at 37±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.

Honey samples to be examined for spores 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 0.01 M PBS pH 7.2 or 0.9% NaCl, transferred to a centrifuge tube and centrifuged at 6000 *g* for 40 minutes. The supernatant is discarded leaving approximately 3 ml per tube that is then vortex-mixed for 1 minute to re-suspend the pellet, and treated as described for larval remains. The samples are high speed vortex-mixed again for 2 minutes and 100–200 µl of the sediment-fluid mixture poured on suitable culture media with the addition of antibiotics and incubate at 37°C for 7–8 days (de Graaf *et al.,* 2013).

Direct plating of diluted honey is widely used, but its sensitivity is inferior to that of the centrifugation method as only a fraction of the total volume will be plated out. Whatever the method of choice is, when honey is analysed quantitatively and threshold values are set, the methodology that was used to establish these values should always be strictly followed.

An aqueous filtrate of pollen can be made by thoroughly dispersing 1 g of pollen in 10 ml final volume sterile distilled water or 0.01 M sodium PBS pH 7.2, and filtering it through Whatman No. 1 paper (de Graaf *et al.,* 2013).

When adult bees are dispatched in ethanol, the latter should be decanted and replaced by sterile water or physiological solution before crushing.

Hornitzky & Karlovskis (1989) developed a culture technique that provides a rapid mean of detecting *P. larvae* spores in adult bees that could act as a source of AFB infection for young larvae. Briefly, each sample of 30 nurse bees is homogenised in 20 ml sterile PBS for 30 seconds. The homogenate is filtered through Whatman No. 1 paper, centrifuged and the pellet is resuspended in PBS. The samples are heat-shocked (see spore-suspension sample treatments) and plated onto a suitable culture medium supplemented with nalidixic acid and pipemidic acid to inhibit the spread of *P. alvei* and other bacteria that can swarm over the plates.

Debris and bee wax (1.5 g) should be dissolved in an organic solvent (10 ml): toluene, chloroform or diethyl ether (de Graaf *et al.,* 2013). The liquid part (2 ml) is then diluted in physiological solution (6 ml). After shaking roughly, this suspension can immediately be plated out (no heat-shock). In another protocol, bee wax is first diluted in water (wax/water 1/10) and heated up to 90°C for 6 minutes. After cooling down, the organic solvent is added (organic solvent/water 1/9) and the mixture is shaken carefully. After 2 minutes standing time, a deposit of a watery solution containing *P. larvae* spores forms.

ii)   Cultivation by Tween 80 Method

One gram of debris or 1 g of wax is put in a test tube with an airtight seal. Larger pieces of wax should be cut by sterile instruments into very small pieces (ideally up to 3 mm in size). Wax pieces contained in debris do not have to be further cut because they are usually very small. The smaller the pieces, the easier and faster is the process of homogenisation. Dry material prepared this way should be stirred thoroughly and diluted with 8.5 ml of sterile distilled water. The resulting suspension is then supplemented with 0.5 ml of Tween 80. Approximately 30 minutes before pipetting, required volume of Tween 80 should be withdrawn from the original container, put into another sterile container with airtight seal and immersed in a hot water bath (70±2°C) to reduce the viscosity of Tween 80 and facilitate its pipetting. The suspension of debris, water and Tween 80 is shaken thoroughly and the test tube is placed in a hot water bath (70±2°C) for 30 minutes. If the wax dissolves slowly or there are pieces of wax larger than 5 mm, the test tube can be left in the water bath for up to 1 hour. While warming the sealed tube in water bath, it should be thoroughly shaken in a longitudinal direction at least three times (preferably in several 5- to 30-second cycles 5– 10 minutes apart). Thorough homogenisation results in development of homogenous greyish brown pulpy material, which can harden as it cools down. Afterwards, tubes are removed from the water bath and allowed to cool down to room temperature, by which time they should be stored for 2–4 hours until a sufficient amount of liquid is separated at the bottom of tubes. Then 2–5 ml of this liquid is withdrawn with a disposable balloon pipette and mixed with the same volume of distilled water in another sterile sealable tube. Again, the resulting mixture should be shaken thoroughly in a longitudinal direction for at least 5 minutes and put into a hot water bath (90±2°C). After 10 minutes, tubes are removed from the bath, allowed to cool down to room temperature and shaken again. Then the material is inoculated in 0.2 ml doses to 3–5 plates of MYPGP with nalidixic acid and at least one plate of blood agar serving as a control. Before culturing, the plates should be dried in a thermostat at 37±1°C. Drying time is selected according to the humidity of the culture medium surface (approximately 30 minutes). Petri dishes must be labelled accurately with the sample identity. The liquid is spread over the plates using a bent sterile plastic/glass stick or the tip of the pipette. The liquid is allowed to dry and the plates are inverted and incubated at 37±1°C for 5–8 days.

1.3.2. Stock solutions (de Graaf *et al.,* 2013)

i) Nalidixic acid stock solution

Prepare by dissolving 0.1 g in 2 ml of 0.1 N NaOH and diluting to 100 ml with 0.01 M phosphate buffer (pH 7.2), (stock concentration 1000 µg/ml). Filter sterilise. Final concentration: 10 µg/ml for larval samples and 20 µg/ml for honey samples.

ii) Pipemidic acid stock

Prepare by dissolving 0.2 g in 2 ml of 0.1 N NaOH and then diluting to 100 ml with 0.01 M phosphate buffer (pH 7.2), (stock concentration 2000 µg/ml). Filter sterilise. Final concentration: 10 µg/ml for honey samples.

After autoclaving and cooling to 50°C add the antibiotics to the media at the final concentration required and pour into sterile Petri plates (20 ml per plate) (de Graaf *et al,* 2013).

1.3.3. Culture media (de Graaf *et al.,* 2013)

Several media for cultivating *P. larvae* have been described but best results were obtained with PLA (*Paenibacillus larvae* agar) (Schuch *et al.*, 2001), MYPGP agar (Dingman & Stahly, 1983), BHIT agar (brain–heart infusion medium supplemented with thiamine), J-agar and CSA (Columbia sheep-blood agar) (Hornitzky & Karlovskis, 1989). The formulations of the media are as follows:

i) PLA (*Paenibacillus larvae* agar)

This selective medium combines three different media to constitute the base, to which antibiotics and egg yolk supplements are added (Schuch *et al.*, 2001). Equal quantities (100 ml) of sterile, molten *Bacillus cereus* selective agar base, trypticase soy agar and supplemented nutrient agar (SNA) are combined and mixed. SNA is composed of (per litre): nutrient agar 23 g, yeast extract 6 g, meat extract 3 g, NaCl 10 g, Na_2HPO_4 2 g; final pH is 7.4 ± 0.2. All solid media are sterilised at 121°C/15 minutes. After the three molten media are combined, 3 ml of stock nalidixic acid, 3 ml of stock pipemidic acid, and 30 ml of 50% egg-yolk suspension are added to form the PLA medium (final concentration 9 µg/ml nalidixic acid and 18 µg/ml pipemidic acid). The PLA medium is poured (20 ml) into sterile Petri dishes and plates are dried before use (45–50°C for 15 minutes).

ii) MYPGP agar (The abbreviation refers to its constituents: Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate)

MYPGP agar is composed of (per litre): Mueller-Hinton broth 10 g, yeast extract 15 g, K_2HPO_4 3 g, glucose 2 g, Na-pyruvate 1 g and agar 20 g (pH 7.1) (Dingman & Stahly, 1983). Addition of nalidixic acid and pipemidic acid is as above.

iii) J-agar

J-agar is composed of (per litre): tryptone 5 g, yeast extract 15 g, K_2HPO_4 3 g, glucose 2 g, agar 20 g (pH 7.3–7.5). Addition of nalidixic acid and pipemidic acid is as above.

iv) CSA (Columbia sheep-blood agar)

CSA is composed of (per litre): 39 g Columbia blood agar base (pH 7.3). After autoclaving and cooling to 50°C, add 5% sterile defibrinated blood (Hornitzky & Karlovskis, 1989). Addition of nalidixic acid and pipemidic acid is as above. Cultivation on CSA slants induces sporulation and allows microscopic detection of the flagellar bundles.

v) BHIT (brain–heart infusion medium supplemented with thiamine) agar

BHIT agar is composed of (per litre): 47 g brain–heart infusion agar (adjusted to pH 6.6 with HCl). After autoclaving and cooling to 50°C, a sterile solution of thiamine hydrochloride is added to obtain a final concentration of 1 mg per litre.

MYPGP agar is routinely used to cultivate *P. larvae* for AFB diagnosis and yielded the highest percentage of spore recovery, while J-agar, BHI and CSA proved to be less efficient in this respect. PLA medium also shows superior plating efficacy and inhibits the majority of micro-organisms normally present in hive and bee products (Schuch *et al.,* 2001).

If cultivation of *P. larvae* is hampered by the occurrence of fungi, the addition of 16.8 μg/ml medium of amphotericin B works very well.

A sterile cotton swab is used to transfer a portion of the sample on to the surface of the solid medium. For a quantitative evaluation, it is recommended to spread a fixed volume of the suspension on the solid agar with a sterile scraper or pipette rather than using cotton swabs.

Inoculated plates are best incubated at $37+1°C$ for 2–4 days in an atmosphere of 5–10% CO_2 in air, although aerobic incubation can also be used. Honey samples should be incubated longer – for at least 6 days and up to 15 days – and checked for suspect colonies at 3 and 6 days.

1.3.4. Identification

i) Colony morphology

Samples from clinically diseased larvae will produce confluent growth on plates after 2–4 days, leading to a subculturing step in order to isolate single colonies.

On PLA, colonies of *P. larvae* are small, pale green to yellow (= the same colour as the medium), with a slightly opaque and rough surface; sometimes the centre is raised.
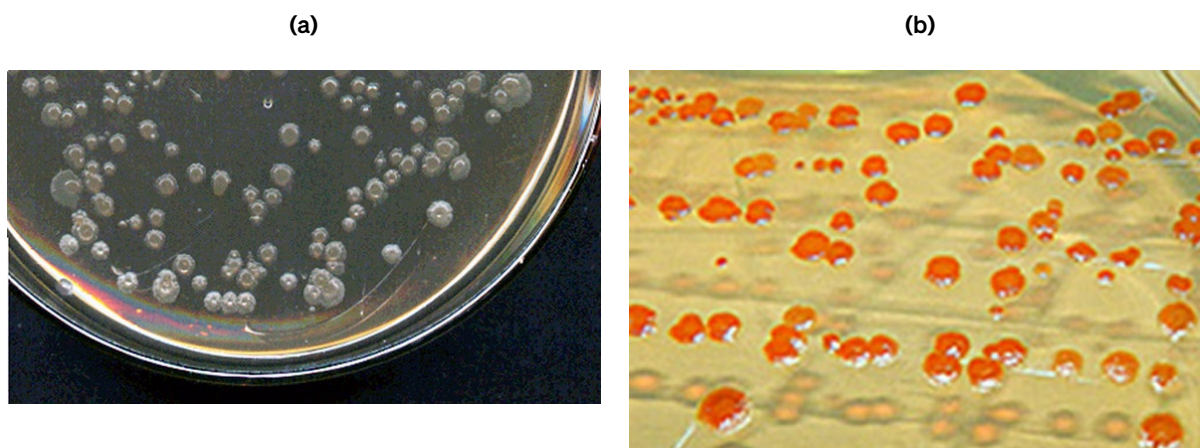
(a)   **(b)**

***Fig.3: Appearance of* Paenibacillus larvae** *colonies cultivated on MYPGP agar plates:*
(*a*) *Reference strain ATCC 9545^T* (*ERIC I*) *and* (*b*) *Strain PL SAG m290* (*ERIC II*). *Photos: A.M. Alippi*

On MYPGP and J-agar, colonies are small, regular, mostly rough, flat or raised and whitish to beige coloured (Figure 3a)

On CSA agar, colonies are small, regular, rough, butyrous and greyish, somewhat transparent and slightly glistening appearance (Figure 4a).

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 CSA (Table 2) (Figures 3b and 4b) (Genersch *et al.*, 2005; Neuendorf *et al.*, 2004). According to Genersch *et al.* (2005) 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.
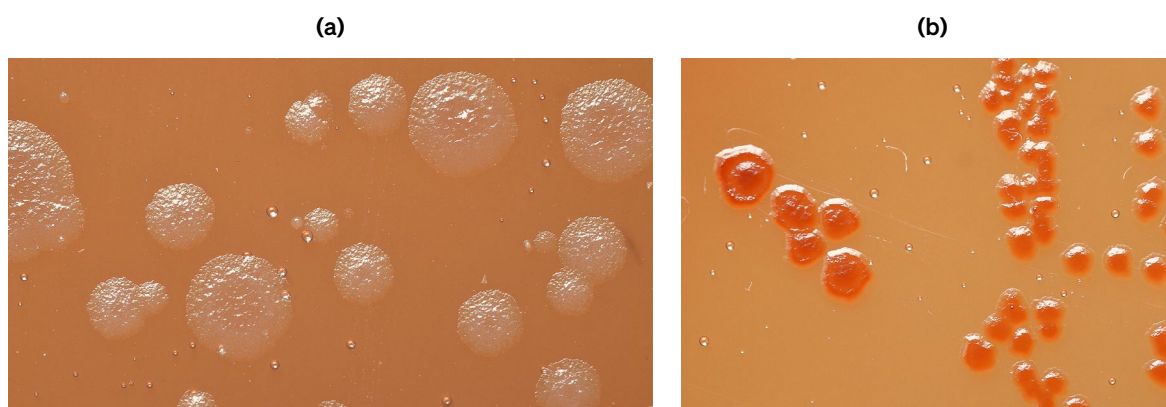
(a)   **(b)**

Fig.4: Characteristic colony morphology of Paenibacillus larvae *cultivated on Columbia sheep blood agar plates:*
(a) Reference strain ATCC 9545^T (ERIC I) and (b) Strain PL SAG m290 (ERIC II). Photos: A.M. Alippi.

It is advised to run *P. larvae* reference strains in parallel, for instance LMG 9820 (other designation: ATCC 9545, DSM 7030, NRRL B-2605, LMG 15969) for the non-pigmented variant and DSM 16115 or DSM 16116 for the pigmented genotype. (For a complete list of *P. larvae* reference strains see de Graaf *et al.,* 2013). The new ERIC V genotype is referenced as strain DSM 106052.

Colony morphology is not conclusive but might serve to select the bacterial colonies for further identification.

Table 2. Phenotypic characteristics of Paenibacillus larvae *ERIC genotypes. All genotypes have ellipsoidal spores*
(data based on Beims et al., 2020 and Genersch et al., 2005)

Characteristic	ERIC I	ERIC II	ERIC III	ERIC IV	ERIC V
Pigmented colonies	–	+	+	Variable	–
Spore surface (as seen by SEM)	Smooth	Convoluted	With ridges	With ridges	With ridges
Growth in nutrient broth	–	+	+	+	+
Fermentation of mannitol	–	+	+	+	NT
Fermentation of salicin	+	–	–	–	NT
Alkaline phosphatase	+	–	+	+	NT
Acid phosphatase	+	–	+	+	NT
Catalase	–	–	Weak, delayed +	Weak, delayed +	NT

NT = not tested

ii)   Polymerase chain reaction

PCR may be used to identify bacterial colonies (= cell/spore suspension) after a cultivation step. DNA can be extracted as follows: one colony is suspended in 50 μl of distilled water and heated to 95°C for 15 minutes. Following centrifugation at 5000 *g* for 5 minutes, 1–5 μl of the supernatant is used as template DNA in a PCR 50 μl mixture (Dobbelaere *et al*., 2001). Commercial DNA extraction kits can also be used, following the manufacturer's instructions for Gram-positive bacteria. See section 1.4.2 below for the PCR method.

iii)   Mass spectrometry

Using a toothpick, a bacterial colony is smeared to two wells of the target plate and allowed to dry at room temperature before 1 μl of matrix (α-cyano-4-hydroxycinnamic acid) is added to each spot and prior to placing the target plate into the machine. MALDI-TOF MS measurement is then performed. The device detects specific mass spectra of bacterial ribosomal peptides. After comparison of detected spectra with the database of known spectra, the bacterial strain is identified (Schafer *et al.,* 2014).

iv)   Biochemical tests

Paenibacillus larvae can also be identified by its biochemical profile. The bacteria are catalase negative or weak delayed positive, they have a typical carbohydrate fermentation profile with acid production from glucose and trehalose, but not from arabinose and xylose, and they can hydrolyse casein or milk. The results of some tests vary within the genotypes, e.g. fermentation of mannitol and salicin (Table 2).

a)   Catalase test

A drop of 3% hydrogen peroxide is placed on an actively growing culture on solid medium. Most aerobic bacteria break down the peroxide to water and oxygen, producing a bubbly foam, but *P. larvae* is negative or weak delayed positive for this reaction, depending on genotype (Table 2) (Haynes, 1972). Organisms can lose their catalase activity with age, resulting in a false negative reaction. When Colombia sheep blood agar is used for cultivation, the test cannot be done on the solid medium, as the presence of sheep blood will cause a false-positive reaction. In this case, colonies should be transferred to a clean microscope slide for the execution of the test. Here the evaluation of the test occurs as above with the naked eye.

b)   Production of acid from carbohydrates

Bacteria are grown in J-broth (per litre: yeast extract 15 g, tryptone 5 g and K_2HPO_4 3 g) in which 0.5% of the test substrate, separately sterilised in aqueous solution, is substituted for the glucose (de Graaf *et al.,* 2013). The carbohydrates used are L (+)-arabinose, D (+)-glucose, D (+)-xylose and D (+)-trehalose. The cultures are tested at 14 days by aseptically removing one ml or less to a spot plate, mixing the sample with a drop of 0.04% alcoholic bromocresol purple, and observing the colour of the indicator. *Paenibacillus larvae* produces acid aerobically from glucose and trehalose. No acid is produced from arabinose and xylose (Alippi, 1992) and variable results are obtained with mannitol and salicin according to the isolate tested (Genersch *et al.,* 2005) (Table 2).

Commercial kits are also available for the biochemical characterisation of *P. larvae* (Neuendorf *et al*., 2004).

c)   Hydrolysis of casein (Schuch *et al.,* 2001)

Casein hydrolysis is assayed using milk agar plus thiamine (per litre: agar 20 g, yeast extract 10 g; sterilised at 121°C/15 minutes). Add to each 70 ml cooled medium 30 ml of UHT (ultra-heat treated) skimmed milk and 1.5 ml filter sterilised 0.1% thiamine solution. Plates are streaked and examined after 5 days of incubation at 36 ± 1°C. *Paenibacillus larvae* hydrolyses casein, hence zones of clearing are observed around bacterial colonies.

v)   Antibody-based techniques (for details refer to de Graaf *et al.,* 2013)

Different antibody-based techniques have been developed for the diagnosis of AFB. Most methods rely on polyclonal rabbit serum developed against pure cultures of *P. larvae* and are used for identification of isolated bacterial colonies or for direct examination of suspicious larval remains.

In an immunodiffusion test the antibodies interact with the bacterial antigen during a double diffusion process, leaving precipitation marks behind. In the fluorescent antibody technique these antibodies are conjugated with a fluorochrome dye. The resulting fluorescent antibody reacts with a bacterial smear on a slide. Any excess antiserum is washed off and the smear is examined by fluorescence microscopy. *Paenibacillus larvae* stains can be recognised specifically as brightly fluorescing bacteria on a dark background. An enzyme-linked immunosorbent assay using a monoclonal antibody specific to *P. larvae* exists. A lateral flow device for rapid confirmation of AFB has been commercialised.
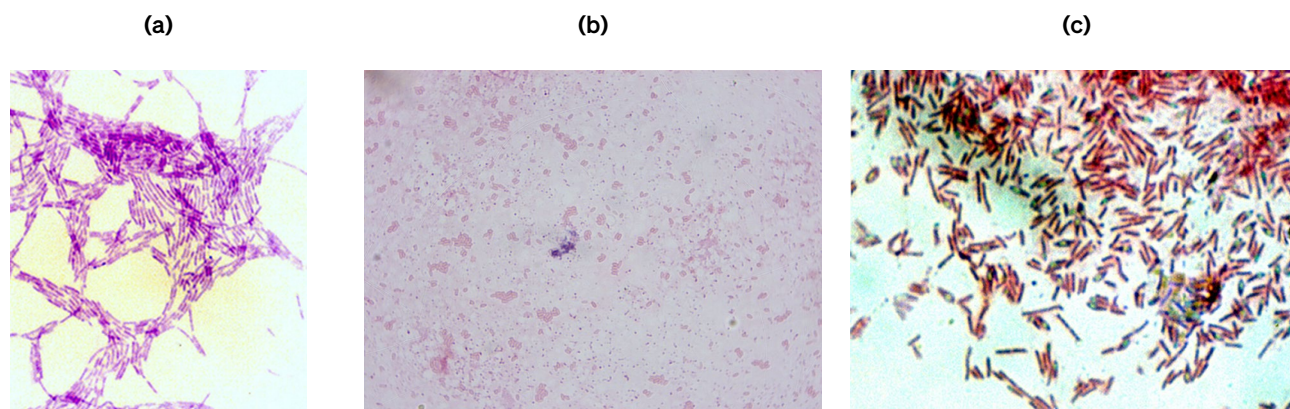
(a)   (b)   (c)

Fig. 5. Microscopic examination of Paenibacillus larvae, *the causative organism of American foulbrood: a) Gram stain reveals Gram-positive rods, occurring singly and in chains (magnification: ×1000); b) Gram stain on diseased larvae reveals pink coloured spores occurring singly or in grapes (magnification: ×1000); c) Schaeffer & Fulton's spore stain reveals that sporangia (stained red) are swollen by the spores (stained green), which are central to terminal (magnification: ×1000). Photos: A.M. Alippi (a and c); J.C. Thomarat & K. Sidi-Boumedine (b).*

vi)   Microscopy

Microscopy and staining are useful for examination of AFB-derived clinical samples or initial bacterial culture. Several methods (including among others Gram staining, etc.) give an indication of the bacteria's structural properties, shape, morphological features, such as cell wall type and presence or absence of endospores.

The most used microscopic techniques are:

a)   **Gram staining**

Gram staining is often done on smears of bacteria from isolated bacterial colonies. *Paenibacillus larvae* appears as Gram-positive rods with slightly rounded ends (Figure 5a).

When the Gram stain is realised on symptomatic larvae smears (Figure 5b), *P. larvae* appears as optically empty pink-coloured ellipsoidal spores (measuring about 0.6 × 1.3 μm). In some cases (recent affection of the larvae) Gram-positive rods can also be observed, either isolated or in more or less long chains.

b)   **Carbol fuchsin staining of larval smears**

When stained with carbol fuchsin, the spores of *P. larvae* (about 0.6 × 1.3 μm) appear ellipsoidal and thick rimmed. Their colour is reddish purple while their centres remain clear.

Note: If the infection appeared in larvae less than 10 days old, long vegetative rods with coalesced flagella will be present. These flagellar bundles are characteristic of the pathogen.

c)   **Schaeffer & Fulton method for staining endospores**

Using this method, the spores appear stained green and the vegetative cells red (Figure 5c). *Paenibacillus larvae* spores are ellipsoidal, central to terminal, swelling the sporangium and thick rimmed, measuring about 0.6 × 1.3 μm.

d)   **Nigrosin negative staining**

A drop of bacterial suspension when mixed with nigrosine solution (5%) leads to a background stained in black whereas *P. larvae* spores appear as bright purplish-red and the vegetative forms as greyish.

As for the other laboratory analyses it is highly important to run appropriate controls, in parallel to the test samples. The different reagents, for bacterial staining, are easily available as ready to use solutions.

1.4. Polymerase chain reaction

1.4.1. Samples for PCR

Cell/spore suspensions and suspensions containing only spores have to be differentiated, the latter requiring a more complex DNA extraction step.

For rapid confirmation of clinical AFB, the samples should be prepared as follows: the remains of two diseased honey bee larvae (= cell/spore suspension) are emulsified in 1 ml of sterile distilled water and mixed thoroughly. 100 µl of this suspension is diluted with 900 µl distilled water. This dilution is vortexed and 100 µl of it is used to extract DNA by heating and centrifugation (see above) (Dobbelaere *et al.*, 2001). Extraction of DNA can also be done using commercial kits according to the manufacturer´s instructions.

All aqueous solutions resulting from the sampling of adult bees, debris, bee wax, pollen and royal jelly should be considered as a spore suspension. Here, the extraction of DNA demands another approach. Indeed, spore suspensions are centrifuged at 6000 *g* and 4°C for 30 minutes. Next, the pellet is subjected to microwave treatment for 5 minutes at maximum power to break the spore coat, and the released DNA is suspended in 30 µl of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA.

When spores are to be detected from honey, DNA is serially diluted with sterile distilled water to eliminate PCR inhibition caused by honey. Another DNA extraction method, based on lysozyme and proteinase K treatment, has been described (Bakonyi *et al.*, 2003). Commercial DNA extraction kits can also be used according to the manufacturer's instructions.

Good results can also be obtained by incubating a pelleted spore suspension in MYPGP broth at 37°C for 2–24 hours. Thereafter, the suspension is centrifuged at 14,500 *g* for 5 minutes, washed with sterile distilled water and resuspended in 200 µl of sterile distilled water. This short incubation step causes spores to germinate, making them sensitive for DNA preparation by heat treatment again (see above) (Lauro *et al.,* 2003).

Positive and negative controls should be run in parallel with the test samples as well as inhibition control. This latter is necessary to detect possible inhibitors of the PCR reaction and thus to avoid a decreased PCR sensitivity or false negative results.

1.4.2. Polymerase chain reaction method

Several conventional PCR protocols have been described to identify *P. larvae* (reviewed by de Graaf *et al.,* 2006 and de Graaf *et al.,* 2013), but two of them, based on the 16S rRNA gene, have proven robustness and are described as follows:

Note: if using commercial PCR mixes the required ingredients may already be included. Check and follow the manufacturer's instructions

PCR reactions (modified from Dobbelaere *et al.*, 2001) are set up as 50 µl mixtures containing:

i)   1–5 µl template DNA (see sample preparation);

ii)   50 pmol forward (AFB-F) and reverse primer (AFB-R);

iii)   10 nmol of each dNTP;

iv)   2 mM $MgCl_2$;

v)   1–2.5 U of *Taq* polymerase in the appropriate PCR buffer containing 2 mM $MgCl_2$.

Reducing the volume of the PCR mixtures to 25 µl is possible.

Use the following PCR conditions: a 95°C (1–15 minutes) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes).

PCR reactions by using PL1 and PL2 primers (Govan *et al.,* 1999) are set up as 50 µl mixtures containing:

i)   1–5 µl template DNA (see sample preparation);

ii)   2 mM MgCl$_2$;

iii)   50 pmol of forward PL1) and reverse (PL2) primer;

iv)   25 mM concentration of each dNTP;

v)   1 U of Taq polymerase per ml.

Reducing the volume of the PCR mixtures to 25 µl is possible.

Use the following PCR conditions: a 95°C (1 minute) step; 30 cycles of 93°C (1 minute), 55°C (30 seconds), and 72°C (1 minute); and a final cycle of 72°C (5 minutes). The molecular weights of the PCR products are determined by electrophoresis in a 0.8% agarose gel and staining with a suitable DNA dye. The PCR products can also be analysed using microfluidics or capillary electrophoresis devices.

Ref.	Name	Sequence (5' → 3')	PCR-product size	Specificity level
(Dobbelaere *et al.,* 2001)	AFB-F AFB-R	CTT-GTG-TTT-CTT-TCG-GGA-GAC-GCC-A TCT-TAG-AGT-GCC-CAC-CTC-TGC-G	1096 bp	species
(Govan *et al.,* 1999)	PL1 PL 2	AAG-TCG-AGC-GGA-CCT-TGT-GTT-TC TCT-ATC-TCA-AAA-CCG-GTC-AGA-GG	973 bp	species

In recent years, real-time PCR methods have been extensively developed as culture-independent approaches to detecting *P. larvae* from various honey and hive samples including larvae and adult honey bees. Compared with conventional PCR, these methods offer the possibility of detecting a target template with its quantification (if needed/necessary) in a robust, highly reproducible, sensitive manner and without post-PCR analysis steps, which are time-consuming and prone to contamination (reviewed by Okamoto *et al.,* 2022).

As in conventional PCR, it is highly important to run appropriate controls (positive, negative as well as inhibition controls) in parallel to the test samples. The use of commercial DNA extraction kits is also possible (to be used according to the manufacturer's instructions).

Regarding the choice of a PCR test for the detection of *P. larvae* samples, users should consider its fitness for purpose. Further advice can be obtained from the WOAH Reference Laboratories[1].

2.   Serological tests

No serological tests are available.

C. REQUIREMENTS FOR VACCINES

No vaccines are available.

ACKNOWLEDGEMENTS

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1   https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

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NB: There are WOAH Reference Laboratories for American foulbrood
(infection of honey bees with *Paenibacillus larvae*) (please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for bee diseases

NB: FIRST ADOPTED IN 1989 AS AMERICAN FOULBROOD DISEASE. MOST RECENT UPDATES ADOPTED IN 2023.