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Diagnosis of American foulbrood in honey bees: a synthesis and proposed analytical protocols

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Summary

Worldwide, American foulbrood (AFB) is the most devastating bacterial disease of the honey bee (*Apis mellifera*). Because the distinction between AFB and powdery scale disease is no longer considered valid, the pathogenic agent has recently been reclassified as one species *Paenibacillus larvae*, eliminating the subspecies designations *Paenibacillus larvae* subsp. *larvae* and *Paenibacillus larvae* subsp. *pulvifaciens*. The creamy or dark brown, glue-like larval remains of infected larvae continue to provide the most obvious clinical symptom of AFB, although it is not conclusive. Several sensitive and selective culture media are available for isolation of this spore-forming bacterium, with the type of samples that may be utilized for detection of the organism being further expanded. PCR methods for identification and genotyping of the pathogen have now been extensively developed. Nevertheless, biochemical profiling, bacteriophage sensitivity, immunotechniques and microscopy of suspect bacterial strains are entirely adequate for routine identification purposes.

Introduction

American foulbrood is an infectious disease of honey bees (*Apis mellifera* and *Apis cerana*) caused by the spore-forming bacterium *Paenibacillus larvae*. It is characterized by the typical 'foul' putrescence emanating from hives containing infected brood and represents a serious, worldwide problem for apiculture (Ellis and Munn 2005). AFB is classified on list B of the Office International des Epizooties (OIE), the World Organization for Animal Health. List B diseases are defined as transmissible diseases which are considered to be of socio-economic and/or public health importance within countries and which are significant in the international trade of animals and animal products.

In the past decade, considerable progress has been made in the understanding and taxonomic reclassification of the causative agent as well as the diagnosis of AFB. Traditional methods such as the recognition of typical clinical symptoms of infection, culture of *P. larvae* from diseased brood, and microscopy provide effective and inexpensive means of diagnosing the disease. In addition, improved detection of *P. larvae* from brood and bee products using molecular techniques, immunotechniques and superior culture techniques have emerged in recent years providing a broader range of methodologies for efficient diagnosis. This review outlines current recommended strategies for the diagnosis of AFB which takes into consideration the reclassification of the causative agent to the single species *P. larvae*.

Pathogenic agent

White (1906) described the species *Bacillus larvae*, the pathogenic agent of AFB. Forty-four years later Katznelson (1950) described a closely related species, *Bacillus pulvifaciens*, also isolated from bee larvae but in association with a rare disease called 'powdery scale'. Both species were transferred to a new genus *Paenibacillus* in 1993 (Ash *et al.* 1993) and later taxonomic revisions described them as being the same species, *P. larvae*, but separated at the sub-species level, i.e. *P. larvae* ssp. *larvae* (*Pll*) (formerly *Bacillus larvae*) and *P. larvae* ssp. *pulvifaciens* (*Plp*) (formerly *Bacillus pulvifaciens*) (Heyndrickx *et al.* 1996).

Genersch *et al.* (2006) proposed to eliminate the sub-species designation based on a polyphasic taxonomic study including colony and spore morphology, biochemical profiles using API systems, SDS-PAGE of whole cell proteins, and rep-PCR fingerprinting and pulsed-field gel electrophoresis (PFGE) of bacterial DNA. In addition, they found that experimental infection with strains identified as *Plp* also leads to the formation of glue-like larval remains that dry down to a hard scale as described for AFB. Moreover, they found that *Plp* strains could be even more virulent with no indication of powdery scale symptoms being observed during their trials. By publishing the proposed reclassification in the official journal of the International Committee on Systematics of Prokaryotes the elimination of the subspecies epithet for *P. larvae* is now validated (Genersch *et al.* 2006). Consequently, *P. larvae* should be considered the full and correct designation for the pathogenic agent of AFB.

Clinical symptoms

Traditionally, the creamy or dark brown glue-like larval remains that can be drawn out as a thread (ropiness) with a matchstick represent the most obvious clinical symptom of AFB (Fig. 1). This symptom is so readily recognized that 'the matchstick test' is often performed by the beekeeper within the apiary and forms part of the first-line warning system against AFB. However, where *P. alvei* is a secondary invader in cases of European foulbrood, clinical signs of infection may look similar to those seen with AFB. This similarity may also involve ropiness of dead brood (Gochnauer *et al.* 1979). Ropy remains of AFB-infected brood are principally from older larvae that die in the upright position, most often after the brood cell has been capped, but larval remains can also be found in open cells. As the infection progresses, cell cappings harbouring dead brood appear greasy, darkened and become concave and punctured with small holes. The larval remains dry down to a flat scale tightly attached to the lower brood cell wall. Very characteristic, but not commonly seen, is the decomposition of the pupal stage in which the tongue protrudes from the head, extending to the top of the brood cell or angling back towards the bottom of the cell. The pupal tongue may also persist in the dried scale.

In addition to the clinical symptoms associated with older larvae and pupae, there is the less noticeable but rapid mortality associated with young larvae, still curled at the base of the uncapped cell. Although these uncapped, infected larvae exhibit no obvious visual symptoms, adult worker bees will readily remove them, leaving only empty cells (Brødsgaard *et al.* 2000). The resulting mottled or spotty appearance of the brood is therefore the result of a varying pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae and empty cells. Similar pathology may also occur with other bee diseases, and should alert the beekeeper to verify which disease agent is present.
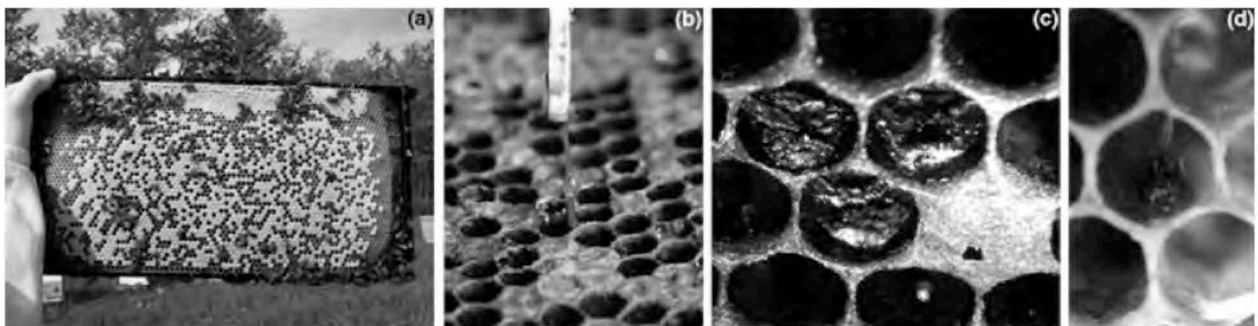

Figure 1 Clinical symptoms of American foulbrood: (a) mottled appearance of the brood often points out a brood disease and should alert the beekeeper. However, similar clinical symptoms may also occur with other diseases and necessitates further examination of the sample. Very characteristic symptoms of American foulbrood are the dark brown glue-like larval remains (b), which dry down to a flat scale (c) and the decomposition of the pupal stage in which the tongue protrudes from the head (d).

Cultivation of *Paenibacillus larvae*

In 100 years of efforts to cultivate *P. larvae*, considerable progress has been made in the development of media promoting spore germination and growth, first attempted with an extract of larval honey bee tissue described by White (1907). Over time, new culture media were developed or adapted for use in AFB diagnosis: J-agar (or J medium) (Gordon *et al.* 1973), brain-heart infusion agar supplemented with thiamine (Gochnauer 1973), Mueller–Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate agar (MYPGP) (Dingman and Stahly 1983), Columbia blood agar (Plagemann 1985), sheep blood agar (Lloyd 1986) and more recently, *Paenibacillus larvae* agar (PLA) (Schuch *et al.* 2001). MYPGP (Nordström and Fries 1995) and PLA (Schuch *et al.* 2001) were shown to have superior plating efficacy. PLA offers the additional advantage that the majority of micro-organisms normally present in the hive and in bee products are inhibited. Moreover, this medium permits incubation in air, whereas previously prescribed media often demanded incubation under CO_2 (Hornitzky and Nicholls 1993).

As *P. larvae* is a spore-forming bacterium, its isolation from biological samples is typically preceded by a heat treatment step. This will significantly reduce the risk that *P. larvae* colonies will become obscured by rapidly growing competitors. Nevertheless, bacteria of the genera *Bacillus*, *Paenibacillus* and *Brevibacillus* may continue to swarm over the plates, most often when samples other than diseased larvae (honey, pollen or adult bees) are cultured. This may necessitate the use of semi-selective media supplemented with nalidixic acid (Hornitzky and Clark 1991) and pipemidic acid (Alippi 1991, 1995).

The type of samples that can be collected for diagnosis of a suspect bee colony has also been further expanded. When the symptoms are obvious, the sampler can cut out a piece of brood comb or collect the larval remains with a swab to be forwarded to the laboratory for further examination. Smears of the remains of diseased larvae for microscopic examination can also be taken in the field (Hornitzky and Wilson 1989). When no symptoms are found, the early detection of spores will identify colonies at risk. Consequently, measures can be taken to prevent the establishment and further dissemination of the disease (de Graaf *et al.* 2001). Apart from brood samples, analysis can be successfully conducted on honey from the brood nest or extracted from the supers (Shimanuki and Knox 1988; Hornitzky and Clark 1991). Samples of pollen (Gochnauer and Corner 1974), wax (Gochnauer 1981), adult workers (Lindström and Fries 2005) or winter hive debris (Titěra and Haklova 2003) can also be used. Routine collection and analysis of samples can be part of an operational or regional AFB detection programme (Schuch *et al.* 2003).

Molecular tools

The first PCR assay for the identification of *P. larvae* was developed by Govan *et al.* (1999) and was based on the 16S rRNA gene. Dobbelaere *et al.* (2001a) described a similar test, though the specificity of the latter was better found and a simplified protocol for the immediate analysis of larval remains was provided. Neither test could differentiate between the former *Pll* and *Plp*, a significant shortcoming during a period in which only *Pll* was considered the causative agent of AFB. Subsequently, several attempts were made to improve the specificity down to the former subspecies level using 16S rRNA-based PCR (Alippi *et al.* 2002; Piccini *et al.* 2002; Kilwinski *et al.* 2004) and others (Alippi *et al.* 2004a; Kilwinski *et al.* 2004; de Graaf *et al.* 2006). With the reclassification of *Pll* and *Plp* into a single species, the scope of these more specific assays have become too narrow for purely diagnostic purposes. A novel nested PCR protocol of Lauro *et al.* (2003) permits direct analysis of honey and hive samples. This test is extremely sensitive to *P. larvae* DNA and might, consequently, identify levels of *P. larvae* that are below those likely to be important for disease.

Aside from PCR, several other molecular tools permit the identification and/or subtyping of the species *P. larvae*. SDS-PAGE of whole cell proteins gives a characteristic profile for *P. larvae* (Hornitzky and Djordjevic 1992) and a subdivision into two clusters (Heyndrickx *et al.* 1996), previously used to discriminate between the previously known subspecies *Pll* and *Plp*. Gas chromatography of methylated fatty acids provides a characteristic peak pattern that allows the identification of *P. larvae* (Drobníková *et al.* 1994; Heyndrickx *et al.* 1996). Several DNA fingerprinting techniques have been performed for epidemiological or taxonomic purposes: RFLP (restriction fragment length polymorphism) (Djordjevic *et al.* 1994), ARDRA (amplified ribosomal DNA restriction analysis), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) (Heyndrickx *et al.* 1996), PFGE (Wu *et al.* 2005) and PCR-based genotyping using primers corresponding to conserved motifs in bacterial repetitive REP (repetitive extragenic palindromic), ERIC (enterobacterial repetitive intergenic consensus) and BOX elements (Alippi and Aguilar 1998a,b). For more information on genotyping and virulence of *P. larvae* we refer to the recent review by Ashiralieva and Genersch (2006).

Biochemical profile

The species *P. larvae* has a characteristic biochemical profile with acid from glucose and trehalose and no acid from arabinose and xylose (Gordon *et al.* 1973). The testing of the carbohydrate acidification profile can be carried out by

using homemade galleries (Gordon *et al.* 1973) or commercialized kits (Carpana *et al.* 1995; Dobbelaere *et al.* 2001b; Neuendorf *et al.* 2004). To our knowledge, only the BIOLOG system has an accompanying software tool that recognizes the *P. larvae*-specific biochemical profile. The result of the catalase test, which is negative for *Pll*, has to be broadened to include the delayed, weak positive reactions of *P. larvae* strains which were previously classified as *Plp* (Heyndrickx *et al.* 1996). The production of high concentrations of proteolytic enzymes during vegetative growth and sporulation of *P. larvae* (reviewed by Chantawanakul and Dancer 2001) has long been utilized for diagnostic purposes in the Holst milk test (Holst 1946). The enzymatic digestion of milk has now been formalized into a casein hydrolysis plate assay (Schuch *et al.* 2001).

Bacteriophage sensitivity

Several bacteriophages have been isolated from *P. larvae* and phage sensitivity has been determined for bacterial strain typing (Drobníková *et al.* 1994; Stahly *et al.* 1999). PPL1c, a virulent mutant bacteriophage isolated from a *P. larvae* field strain, lyses nearly all former *Pll* strains (97–99%; Stahly *et al.* 1999; Alippi *et al.* 2004b). As species from the genera *Bacillus*, *Brevibacillus* and *Paenibacillus* (other than *P. larvae*) were proved to be resistant to PPL1c-induced lysis, phage sensitivity to PPLc1 offers an interesting supplementary tool for *P. larvae* identification (Fig. 2) (Stahly *et al.* 1999).

Immunotechniques

Several different antibody-based techniques have been developed for diagnosis of AFB. These include the immunodiffusion test (Peng and Peng 1979), the immunofluorescence assay (Otte 1973), where fluorochrome-conjugated polyclonal antibodies are used to visualize specific bacteria (Toshkov *et al.* 1970; Zhavnenko 1971), and the enzyme-linked immunosorbent assay using monoclonal antibodies (Olsen *et al.* 1990). Recently, a lateral flow device test kit for AFB detection has been developed (Vita, Hants, UK). It allows rapid confirmatory diagnosis in the field and is now commercially available. The utility of each of these diagnostic tests is highly dependent on the specificity of the polyclonal or monoclonal antibody preparations used. Cross-recognition of closely related bacteria should be avoided.

Microscopy

The diagnosis of AFB by microscopic examination of carbol fuchsin-stained smears of dead larvae is entirely satisfactory (Hornitzky and Wilson 1989). The test is based
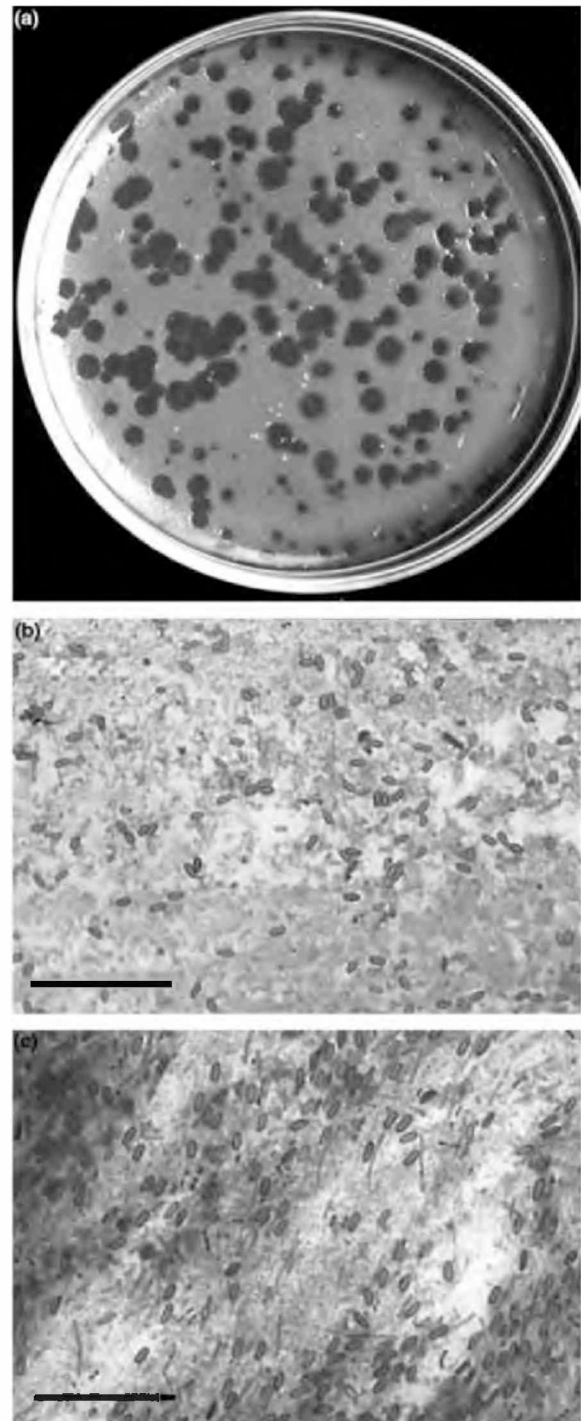

Figure 2 Diagnosis of American foulbrood: nearly all *Paenibacillus larvae* strains are sensitivity to PPL1c, a virulent mutant bacteriophage, resulting in plaque formation in a simple phage sensitivity test (a). Microscopic examination of carbol fuchsin-stained smears of larval remains permits rapid confirmation of clinically diseased larvae, based on the morphology of the spores of *Paenibacillus larvae* (b) (bar = 10 μm); spores of *Paenibacillus alvei* (c) are larger and stain more deeply.

Table 1 Different techniques to identify *Paenibacillus larvae*

Technique	Principle	Samples	Advantages	Disadvantages
Cultivation	Germination and growth of *Paenibacillus larvae* spores on solid medium	Brood, honey, adult bees, pollen, wax, hive debris	Detection of *P. larvae* in bee products facilitates tracing infection sources; Very suitable for American foulbrood (AFB) detection programmes; Permits quantification of the spore-load; Allows to test spore viability	Requires an additional identification step of suspect *P. larvae* colonies; Semi-selective media usually required to avoid contamination with other bacteria
Biochemical profiling	Identification of the species of bacteria based on the carbohydrate acidification profile, the catalase test and the casein hydrolysis plate test	Bacterial colonies	Is a traditional microbiological approach that can be performed in most microbiology laboratories	Requires a first step of isolation and cultivation of bacteria; Results for a full profile are available after 2 to 3 weeks, although identification as *P. larvae* can be based on Gram reaction, catalase reaction and colony morphology
Phage sensitivity test	Plaque formation in a semi-solid medium as a result of bacterial cell lysis	Bacterial colonies	Easy and simple test to perform; Rapid diagnosis of AFB; Low cost	Requires a first step of isolation and cultivation of bacteria
PCR	Amplification of specific bacterial DNA using a single primer set	Bacterial colonies, brood, honey	Permits rapid confirmation without cultivation step starting from diseased brood	Needs sophisticated equipment
Nested PCR	Amplification of specific bacterial DNA by using external and internal primer sets in subsequent reactions	Brood, honey, adult bees	Permits rapid confirmation starting from a broad range of samples; Because of its high sensitivity very suitable for AFB detection programmes	Needs sophisticated equipment; Can identify levels of *P. larvae* that are below those likely to be important for disease; Can identify the presence of dead spores or spores that fail to germinate, both not important for disease; Nested PCRs are more prone to contamination
Microscopy	Morphological identification of *P. larvae* spores	Brood	Smears can be made in the field and forwarded to the laboratory for examination; Rapid diagnosis of AFB; Low cost	Only for confirmation of clinically diseased larvae
Immunotechniques	Different tools for identification based on specific antigen–antibody interactions	Brood	The commercialized lateral flow device permits easy confirmation of clinical AFB in the field; Rapid diagnosis of AFB	Utility of each test is highly dependent on the specificity of the antibody preparation used

on detecting *P. larvae* spores. The spores of *P. alvei* – an important secondary invader in cases of European foulbrood which may cause AFB-like symptoms (see above) – can be differentiated morphologically from *P. larvae* spores because they are larger (about 2·0 × 0·8 μm) and stain more deeply with carbol fuchsin than *P. larvae* spores which are 1·3 × 0·6 μm (Fig. 2) (Hornitzky and Wilson 1989).

Conclusions and future prospects

With the new designation of the pathogenic agent of AFB, an important obstacle for the diagnosis of AFB has been eliminated. Molecular biological techniques have become increasingly accurate and sensitive for identifying *P. larvae* in honey bees and hive materials (Table 1). Nevertheless, biochemical profiling and bacteriophage sensitivity of suspect bacterial strains, microscopic examinations and immunotechniques provide entirely adequate alternatives (Table 1).

In the future, it may also be possible to expand genetically based diagnoses to include specific traits of interest to *P. larvae* researchers and regulators (e.g. antibiotic resistance, virulence and geographical source). With funding from the U.S. Department of Agriculture, an effort to sequence, assemble and annotate the *P. larvae* genome has been ongoing since 2004 (http://www.hgsc.bcm.tmc.edu/projects/microbial/). This project is providing numerous candidates for genes involved in *P. larvae* pathogenesis (Evans and Pettis 2005), along with polymorphic regions that can be used to develop new standardized tests for intraspecific boundaries. Having a genome sequence in hand should be especially helpful for identifying specific traits that make this such a virulent pathogenic agent and that regulate its activation during infection.

References

Alippi, A.M. (1991) A comparison of laboratory techniques for the detection of significant bacteria of the honey bee, *Apis mellifera*, in Argentina. *J Apic Res* **30**, 75–80.

Alippi, A.M. (1995) Detection of *Bacillus larvae* spores in Argentinian honeys by using a semi-selective medium. *Microbiologia* **11**, 343–350.

Alippi, A.M. and Aguilar, O.M. (1998a) Unique DNA fingerprint patterns of *Paenibacillus larvae* subsp. *larvae* strains. *J Apic Res* **37**, 273–280.

Alippi, A.M. and Aguilar, O.M. (1998b) Characterization of isolates of *Paenibacillus larvae* subsp. *larvae* from diverse geographical origin by the polymerase chain reaction and BOX primers. *J Invertebr Pathol* **72**, 21–27.

Alippi, A.M., López, A.C. and Aguilar, O.M. (2002) Differentiation of *Paenibacillus larvae* subsp. *larvae*, the cause of

American foulbrood of honeybees, by using PCR and restriction fragment analysis of genes encoding 16S rRNA. *Appl Environ Microbiol* **68**, 3655–3660.

Alippi, A.M., López, A.C. and Aguilar, O.M. (2004a) A PCR-based method that permits specific detection of *Paenibacillus larvae* subsp. *larvae*, the cause of American foulbrood of honey bees, at the subspecies level. *Lett Appl Microbiol* **39**, 25–33.

Alippi, A.M., Reyaldi, F.J., López, A.C., De Giusti, M.R. and Aguilar O.M. (2004b) Molecular epidemiology of *Paenibacillus larvae larvae* and incidence of American foulbrood in Argentinean honeys from Buenos Aires province. *J Apic Res* **43**, 135–143.

Ash, C., Priest, F.G. and Collins, M.D. (1993) Molecular identification of ribosomal RNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test – proposal for the creation of a new genus *Paenibacillus*. *Antonie Van Leeuwenhoek* **64**, 253–260.

Ashiralieva, A. and Genersch, E. (2006) Reclassification, genotypes and virulence of *Paenibacillus larvae*, the etiological agent of American foulbrood in honeybees – a review. *Apidologie* **37**, 411–420.

Brødsgaard, C.J., Hansen, H. and Ritter, W. (2000) Progress of *Paenibacillus larvae larvae* infection in individually inoculated honey bee larvae reared singly *in vitro*, in micro colonies, or in full-size colonies. *J Apic Res* **39**, 19–27.

Carpana, E., Marocchi, L. and Gelmini, L. (1995) Evaluation of the API 50CHB system for the identification and biochemical characterization of *Bacillus larvae*. *Apidologie* **26**, 11–16.

Chantawanakul, P. and Dancer, B.N. (2001) American foulbrood in honey bees. *Bee World* **83**, 168–180.

Dingman, D.W. and Stahly, D.P. (1983) Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. *Appl Environ Microbiol* **46**, 860–869.

Djordjevic, S., Ho-Shon, M. and Hornitzky, M. (1994) DNA restriction endonuclease profiles and typing of geographically diverse isolates of *Bacillus larvae*. *J Apic Res* **33**, 95–103.

Dobbelaere, W., de Graaf, D.C., Peeters, J.E. and Jacobs, F.J. (2001a) Development of a fast and reliable diagnostic method for American foulbrood disease (*Paenibacillus larvae* subsp. *larvae*) using a 16S rRNA gene based PCR. *Apidologie* **32**, 363–370.

Dobbelaere, W., de Graaf, D.C., Peeters, J.E. and Jacobs, F.J. (2001b) Comparison of two commercial kits for biochemical characterization of *Paenibacillus larvae larvae* in the diagnosis of AFB. *J Apic Res* **40**, 37–40.

Drobníková, V., Richter, V., Häusler, J. and Pytelová, I. (1994) Characterization of *Bacillus larvae* and related bacilli by chromatography of cell fatty acids. *J Apic Res* **33**, 69–74.

Ellis, J.D. and Munn, P.A. (2005) The worldwide health status of honey bees. *Bee World* **86**, 88–101.

Evans, J.D., Pettis, J.S. (2005) Colony-level impacts of immune responsiveness in honey bees, *Apis mellifera*. *Evolution* **59**, 2270–2274.

Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J. and Fries, I. (2006) Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. *Int J Syst Evol Microbiol* **56**, 501–511.

Gochnauer, T.A. (1973) Growth, protease formation, and sporulation of *Bacillus larvae* in aerated broth culture. *J Invertebr Pathol* **22**, 251–257.

Gochnauer, T.A. (1981) The distribution of *Bacillus larvae* spores in the environs of colonies infected with American foulbrood disease. *Am Bee J* **121**, 332–335.

Gochnauer, T.A. and Corner, J. (1974) Detection and identification of *Bacillus larvae* in a commercial sample of bee-collected pollen. *J Apic Res* **13**, 265–267.

Gochnauer, T.A., Furgala, B. and Shimanuki, H. (1979) Diseases and enemies of the honey bee. In *The Hive and the Honey Bee* ed. Graham, J.M. pp. 617–619. Hamilton, IL: Dadant & Sons, Inc.

Gordon, R.E., Haynes, W.C. and Pang, H.N. (1973) *The Genus Bacillus*. Agriculture Handbook No. 427. Washington, DC: U.S. Department of Agriculture.

Govan, V.A., Allsopp, M.H. and Davison, S. (1999) A PCR detection method for rapid identification of *Paenibacillus larvae*. *Appl Environ Microbiol* **65**, 2243–2245.

de Graaf, D.C., Vandekerchove, D., Dobbelaere, W., Peeters, J.E. and Jacobs, F.J. (2001) Influence of the proximity of American foulbrood cases and apicultural management on the prevalence of *Paenibacillus larvae* spores in Belgian honey. *Apidologie* **32**, 587–599.

de Graaf, D.C., De Vos, P., Heyndrickx, M., Van Trappen, S., Peiren, N. and Jacobs, F.J. (2006) Identification of *Paenibacillus larvae* to the subspecies level: an obstacle for AFB diagnosis. *J Invertebr Pathol* **91**, 115–123.

Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P., Logan, N.A., Ali, N. *et al.* (1996) Reclassification of *Paenibacillus* (formerly *Bacillus*) *pulvifaciens* (Nakamura 1984) Ash et al 1994, a later subjective synonym of *Paenibacillus* (formerly *Bacillus*) *larvae* (White 1906) Ash et al 1994, as a subspecies of *P. larvae*, with emended descriptions of *P. larvae* as *P. larvae* subsp *larvae* and *P. larvae* subsp *pulvifaciens*. *Int J Syst Bacteriol* **46**, 270–279.

Holst, E.C. (1946) A simple field test for American foulbrood. *Am Bee J* **86**, 14–34.

Hornitzky, M.A.Z. and Clark, S. (1991) Culture of *Bacillus larvae* from bulk honey samples for the detection of American foulbrood. *J Apic Res* **30**, 13–16.

Hornitzky, M.A.Z. and Djordjevic, S.P. (1992) Sodium dodecyl sulphate-polyacrylamide profiles and Western blots of *Bacillus larvae*. *J Apic Res* **31**, 47–49.

Hornitzky, M.A.Z. and Nicholls, P.J. (1993) J-medium is superior to sheep blood agar and brain heart infusion agar for the isolation of *Bacillus larvae* from honey samples. *J Apic Res* **32**, 51–52.

Hornitzky, M.A.Z. and Wilson, S.C. (1989) A system for the diagnosis of the major bacterial brood diseases of honeybees. *J Apic Res* **28**, 191–195.

Katznelson, H. (1950) *Bacillus pulvifaciens* (n. sp.), an organism associated with powdery scale of honeybee larvae. *J Bacteriol* **59**, 153–155.

Kilwinski, J., Peters, M., Ashiralieva, A. and Genersch, E. (2004) Proposal to reclassify *Paenibacillus* larvae subsp. *pulvifaciens* DSM 3615 (ATCC 49843) as *Paenibacillus larvae* subsp. *larvae*. Results of a comparative biochemical and genetic study. *Vet Microbiol* **104**, 31–42.

Lauro, F.M., Favaretto, M., Covolo, L., Rassu, M. and Bertoloni, G. (2003) Rapid detection of *Paenibacillus larvae* from honey and hive samples with a novel nested PCR protocol. *Int J Food Microbiol* **81**, 195–201.

Lindström, A. and Fries, I. (2005) Sampling of adult bees for detection of American foulbrood (*Paenibacillus larvae* subsp. *larvae*) spores in honey bee (*Apis mellifera*) colonies. *J Apic Res* **44**, 82–86.

Lloyd, J.M. (1986) Simplified laboratory diagnosis of American foulbrood disease. *J Apic Res* **25**, 55–57.

Neuendorf, S., Hedtke, K., Tangen, G. and Genersch, E. (2004) Biochemical characterization of different genotypes of *Paenibacillus larvae* subsp. *larvae*, a honey bee bacterial pathogen. *Microbiology* **150**, 2381–2390.

Nordström, S. and Fries, I. (1995) A comparison of media and cultural conditions for identification of *Bacillus larvae* in honey. *J Apic Res* **34**, 97–103.

Olsen, P.E., Grant, G.A., Nelson, D.L. and Rice, W.A. (1990) Detection of American foulbrood disease of the honeybee, using a monoclonal antibody specific to *Bacillus larvae* in an enzyme-linked immunosorbent assay. *Can J Microbiol* **36**, 732–735.

Otte, E. (1973) Contribution to the laboratory diagnosis of American foulbrood (AFB) of the honey bee with particular reference to the fluorescent antibody technique. *Apidologie* **4**, 331–339.

Peng, Y.S. and Peng, K.Y. (1979) A study on the possible utilization of immunodiffusion and immunofluorescence techniques as the diagnostic methods for American foulbrood of honeybees (*Apis mellifera*). *J Invertebr Pathol* **33**, 284–289.

Piccini, C., D'Alessandro, B., Antúnez, K. and Zunino, P. (2002) Detection of *Paenibacillus larvae* subspecies *larvae* spores in naturally infected bee larvae and artificially contaminated honey by PCR. *World J Microbiol Biotechnol* **18**, 761–765.

Plagemann, O. (1985) A simple culture method for the bacteriological identification of *Bacillus larvae* on Columbia blood-slant agar. *Berl Munch Tierarztl Wochenschr* **98**, 61–62.

Schuch, D.M.T., Madden, R.H. and Sattler, A. (2001) An improved method for the detection and presumptive identification of *Paenibacillus larvae* subsp *larvae* spores in honey. *J Apic Res* **40**, 59–64.

Schuch, D.M.T., Tochetto, L.G. and Sattler, A. (2003) Isolamento de esporos de *Paenibacillus larvae* subsp. *larvae* no Brasil. *Pesq Agropec Bras* **38**, 353–354.

Shimanuki, H. and Knox, D.A. (1988) Improved method for the detection of *Bacillus larvae* spores in honey. *Am Bee J* **128**, 353–354.

Stahly, D.P., Alippi, A.M., Bakhiet, N., Campana, C.F., Novak, C.C. and Cox, R. (1999) PPL1c, a virulent mutant bacteriophage useful for identification of *Paenibacillus larvae* subspecies *larvae*. *J Invertebr Pathol* **74**, 295–296.

Titěra, D. and Haklova, M. (2003) Detection method of *Paenibacillus larvae larvae* from beehive winter debris. *Apiacta* **38**, 131–133.

Toshkov, A., Valarianov, T. and Tomov, A. (1970) The immunofluorescence method and the quick and specific diagnosis of American foulbrood of beebrood. *Bull Apic* **13**, 13–18.

White, G.F. (1906) *The Bacteria of the Apiary, with Special Reference to Bee Diseases*. Technical Series No. 14. Washington, DC: U.S. Department of Agriculture, Bureau of Entomology.

White, G.F. (1907) *The Cause of American Foulbrood*. Circular 94. Washington, DC: U.S. Department of Agriculture, Bureau of Entomology.

Wu, X.Y., Chin, J., Ghalayini A. and Hornitzky M. (2005) Pulsed-field gel electrophoresis typing and oxytetracycline sensitivity of *Paenibacillus larvae* subsp. *larvae* isolates of Australian origin and those recovered from honey imported from Argentina. *J Apic Res* **44**, 87–92.

Zhavnenko, V.M. (1971) Indirect method of immunofluorescence in the diagnosis of foulbrood (American and European). *Veterinariia* **8**, 109–111.