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Paenibacillus larvae and American Foulbrood – long since known and still surprising

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Abstract: American foulbrood (AFB) is one of the most deleterious bacterial honey bee diseases though affecting only the larval stages of bees. The causative agent of AFB is the Gram-positive bacterium *Paenibacillus larvae* firstly described in the beginning of the 20th century. Since then AFB has become one of the best-studied honey bee diseases. However many aspects of AFB and the related pathogen *P. larvae* still remain elusive. This review will focus on three main topics by reviewing both, historical data and new results obtained with modern laboratory techniques: (i) *P. larvae* and the pathogenesis of AFB, (ii) transmission of *P. larvae* spores and AFB, and (iii) diagnosis of AFB.

Zusammenfassung: Die Amerikanische Faulbrut (AFB) gehört zu den gefährlichsten bakteriellen Bruterkrankungen der Honigbienen. Der Verursacher der AFB ist das Gram-positive Bakterium *Paenibacillus larvae*, welches erstmals zu Beginn des 20. Jahrhunderts beschrieben wurde. Seitdem ist die AFB eine der am besten erforschten Bienenkrankheiten. Trotzdem sind immer noch viele Aspekte der AFB und des dazugehörenden Pathogens *P. larvae* unerforscht. Dieser Übersichtsartikel wird sich auf drei Hauptthemen beschränken und hierzu jeweils sowohl historische Daten als auch neueste Ergebnisse vorstellen, die mit modernen Labortechniken erarbeiten wurden: (i) *P. larvae* und Pathogenese der AFB, (ii) Übertragung der *P. larvae*-Sporen und der AFB und schließlich (iii) Diagnose der AFB.

1. Introduction

Honeybees are important pollinators of crops, fruit and wild flowers. Therefore, they are indispensable for a sustainable and profitable agriculture but also for the maintenance of non-agricultural ecosystems. Honeybees are attacked by numerous pathogens including viruses, bacteria, fungi and parasites. Prior to the arrival of *Varroa destructor* (Boecking and Genersch, 2008) the most economically important diseases of honey bees were the bacterial diseases European foulbrood and American foulbrood. As their names suggest, they only infect the larval stage of honey bees. American foulbrood (AFB) is still one of the most deleterious bee diseases not only able to kill infected individuals but also to be potentially lethal to infected colonies. Spreading of the disease both locally and globally is facilitated by beekeeping practice like exchanging hive and bee material between colonies, managing numerous hives in a confined area and the trading of queens, colonies ("package bees") and honey. Meanwhile, AFB has spread worldwide. In many countries, AFB is a notifiable disease and measures are often regulated by corresponding laws. Most authorities consider burning of diseased colonies and contaminated hive material the only workable control measure. Thus, AFB is causing considerable economic loss to beekeepers all over the world. This review will both, cover the historical development as well as the recent advances in AFB research.

2. American foulbrood and its etiological agent

The history of AFB presumably dates back to Aristotle (384–322 b. C.) who described in book IX of his History of Animals a diseased condition which "is indicated in a lassitude on the part of the bees and in malodorousness of the hive". However, although Aristotle's description is not sufficient to identify American foulbrood with certainty it makes clear that bees then were much the same as now and diseases we now call foulbrood (American or European foulbrood) probably already existed in antiquity. In the 18th century then, the Saxon naturalist Schirach described a honeybee disease which he called 'foulbrood' and which was characterized by a foul smell coming from the diseased colony (Schirach, 1769). About a cen-

tury later, two different etiopathologies of 'foulbrood' were described: 'mild and curable' of unsealed brood (most likely what we now call European foulbrood), and 'malignant and uncurable' (almost certainly American foulbrood) (Dzierzon, 1882). In 1885, *Bacillus alvei* was suspected to be the causative agent of 'foulbrood' (Cheshire and Cheyne, 1885). When in 1906 the American scientist White failed to isolate *Bacillus alvei* from foulbrood diseased larvae but instead isolated what he called *Bacillus larvae* (White, 1906) it became evident that there were actually two different bacterial brood diseases to which the name 'foulbrood' had been applied: On the one hand a disease now known as European foulbrood (EFB) caused by *Melissococcus plutonius* with *Bacillus alvei* as a frequent secondary invader (Bailey, 1957 and 1983); on the other hand what was then called American foulbrood (AFB) with *Bacillus larvae* isolated as etiological agent (White, 1906).

After its first description in 1906, the etiological agent of AFB was reclassified several times and can therefore be found in the literature as *Bacillus larvae* (White, 1906) with a close relative causing the so-called 'powdery scale disease' named *Bacillus pulvifaciens* (Katznelson, 1950), as two separate species *Paenibacillus larvae* and *Paenibacillus pulvifaciens* (Ash et al., 1991 and 1993), and as two subspecies *Paenibacillus larvae larvae* and *Paenibacillus larvae pulvifaciens* (Heyndrickx et al., 1996). The final revision of the classification within the species *Paenibacillus larvae* (Genersch et al., 2006) resulted in the reclassification of the subspecies *P. l. larvae* and *P. l. pulvifaciens* (Heyndrickx et al., 1996) as one species *P. larvae* without subspecies differentiation. Experimental laboratory infections of honeybee larvae with a wide range of representatives of the species *P. larvae* including different reference strains of '*P. l. pulvifaciens*' had clearly demonstrated that all tested *P. larvae* strains caused the characteristic disease symptoms of American foulbrood in infected larvae with dead larvae developing into a ropy mass and drying down to a hard scale (Genersch et al., 2005 and 2006).

P. larvae is a rod-shaped, Gram-positive bacterium about 2.5–5 μm by 0.5–0.8 μm which is motile due to its peritrichous flagellae. *P. larvae* is able to form extremely tenacious endospores which are the only infectious form of this organism. Larvae become infected by swallowing spores that contaminate their food. During the first 12–36 hours after hatching larvae are most susceptible to infection. Recently, it was demonstrated that spore contaminated honey can serve as an environmental reservoir of the infectious stages of *P. larvae* (Lindström et al., 2008a).

3. Pathogenesis

The pathogenic mechanism was originally thought to be through the growth of *P. larvae* in the organ cavity of honey bee larvae (Ritter, 1994 and 1996, and references therein). The accepted view was that the bacteria germinate preferentially at either end of the midgut of honey bee larvae soon after entering the larval midgut, then move through the gut epithelium by phagocytosis (Davidson, 1973) into the haemocoel, the presumed primary place of bacterial proliferation (Bailey and Ball, 1991). However, recent studies using fluorescence *in situ* hybridization (FISH) and a *P. larvae* specific 16S rRNA probe demonstrated that *P. larvae* spores germinate at any place in the larval midgut and that vegetative *P. larvae* bacteria colonize the larval midgut where they massively proliferate living like commensals from the food ingested by the larvae. Eventually the honey bee larvae gut contains nothing but these pathogenic bacteria. It is not until then that the bacteria penetrate the midgut epithelium and 'burst' out of the gut into the organ cavity thereby killing the larvae (Yue et al., 2008). In contrast to earlier studies suggesting that penetration of the midgut epithelium by *P. larvae* occurs *via* phagocytosis (Davidson, 1973; Gregorc and Bowen, 1998) FISH analysis revealed that *P. larvae* rather used the paracellular route for crossing the midgut epithelium and entering the haemocoel (Yue et al., 2008). Numerous studies in the past had shown that *P. larvae* secretes highly active extracellular proteases during the process of infection (Holst and Sturtevant, 1940; Holst, 1946; Dancer and Chantawannakul, 1997). However, the function of these proteases remained elusive (Chantawannakul and Dancer, 2001). The data presented by Yue and co-workers (Yue et al., 2008) indicate that at least some of these proteases might be responsible for the disruption of the epithelial barrier integrity by degrading the proteins forming the cell-cell and cell-matrix junctional structures thereby allowing *P. larvae* to invade the haemocoel and killing the larva.

After the infected larvae have died from AFB the bacteria continue their destructive work by degrading the larval remains to a brownish, semi-fluid, glue-like colloid (ropy stage). Later still this tissue detritus dries down to a hard scale (foulbrood scale) tightly adhering to the lower cell wall. These scales contain millions of spores, are highly infectious, and contribute to disease transmission within and between colonies (Sturtevant, 1932; Bailey and Ball, 1991; Gregorc and Bowen, 1998; Lindström et al., 2008a).

4. Virulence and transmission within colonies

The species *P. larvae* can be subdivided into four different genotypes based on rep-PCR (Versalovic et al., 1994) using ERIC-primers (Genersch and Otten, 2003; Genersch et al., 2006). These genotypes were designated ERIC I to ERIC IV (Genersch et al., 2006). Epidemiological studies on the incidence and prevalence of these genotypes are still scarce. There are a few studies available from European countries (Genersch and Otten, 2003; Genersch et al., 2006; Peters et al., 2006; Loncaric et al., 2008) showing that ERIC I and ERIC II are frequently isolated from foulbrood diseased colonies at least in Germany, Austria, Sweden, and Finland whereas ERIC III/IV are absent in these countries. From the American continent even less is known on the epidemiology of *P. larvae* but obviously ERIC I is rather common whereas ERIC II as well as ERIC III/IV could not be identified in field isolates in recent years so far (Alippi et al., 2004; Antunez et al., 2007). The four ERIC genotypes differ in spore and colony morphology, in their metabolic fingerprints, SDS-PAGE profiles, and pulsed field-gel electrophoresis (PFGE) patterns (Neuendorf et al., 2004; Ge-

Tab. 1 Characteristics of the relevant *P. larvae*-genotypes ERIC I and II.

	Paenibacillus larvae	
Genotypes acc. to ERIC-PCR	ERIC I	ERIC II
Isolated from AFB outbreaks in Europe	yes	yes
LT_{100} in exposure bioassays	~ 12 d	~ 7 d
Proportion of larvae dying after pupation in exposure bioassays	40% – 25%	< 10%
Clinical diagnosis based on ropy mass / foulbrood scales	adequate	impaired
Germination on nutrient agar after heat pre-treatment of samples	stimulated	inhibited
Laboratory detection of spores acc. to standard procedures	adequate	impaired

nersch et al., 2006). In addition and more importantly, they differ in virulence (Genersch et al., 2005 and 2006).

Recent studies addressing these differences in virulence within the species *P. larvae* using exposure bioassays to experimentally infect young, susceptible larvae in the laboratory demonstrated genotype-specific differences in the etiopathology of AFB, as revealed by differences in the LT_{100} (lethal time it takes a pathogen to kill 100% of the infected individuals) of the tested representatives of all four genotypes of *P. larvae* (Genersch et al., 2005 and 2006). Of special clinical importance are the observed differences between ERIC I and ERIC II since these are the genotypes causing AFB outbreaks in Europe (Tab. 1). Due to a rather fast disease progression in larvae infected by *P. larvae* ERIC II all infected individuals were dead by day six or seven post infection resulting in less than 10% of the infected larvae dying after the onset of metamorphosis (i.e. time point of cell capping in the colony) while around 90% were killed already before 'cell capping'. These larvae could easily be detected and removed by the nurse bees under natural conditions and ropy mass and foulbrood scales would develop only occasionally resulting in an impaired disease transmission within the colony. Larvae infected by *P. larvae* ERIC I showed a slower disease progression. It took the pathogen up to twelve days post infection to kill all infected larvae resulting in around 40 – 25% of the infected larvae dying after the onset of metamorphosis, i.e. cell capping in the colony. Larvae dying after cell capping are hardly detected and removed by nurse bees under natural conditions. Therefore, they will have the chance to develop into a ropy mass and a highly infectious foulbrood scale containing millions of spores which facilitate and drive disease transmission within the colony. These results on and interpretations of virulence differences between *P. larvae* ERIC I and II suggest that an infection caused by ERIC I will rapidly spread within the colony and colony collapse will develop rather fast. In contrast, an infection caused by ERIC II will spread more slowly within the colony and the pathogen might need more than one season to weaken the colony and cause colony collapse (Ashiralieva and Genersch, 2006; Genersch, 2007). At the same time the typical clinical symptoms of an AFB infection (ropy mass and foulbrood scale) will be obscured in a colony infected by *P. larvae* ERIC II due to the hygienic behavior of the bees (Tab. 1).

5. Transmission between colonies

Understanding *P. larvae* transmission within a colony is important to predict the fate of an infected colony and the time span between infection and occurrence of clinical symptoms or colony collapse. Knowledge about the transmission of *P. larvae* between colonies is necessary to understand the epidemiology of AFB and to evaluate the expected spread of the pathogen within an apiary and between apiaries. While most diseases of honey bees are mainly vertically transmitted, AFB was considered the paradigm of a horizontally transmitted bee disease (Fries and Camazine, 2001). This picture is now changing with reports on the vertical transmission of *P. larvae* spores between colonies through swarming with subsequent disease outbreaks (Fries et al., 2006).

Horizontal transmission of *P. larvae* between colonies occurs mainly through robbing and drifting bees. Robbing bees are foragers from one colony which invade another colony to steal honey. Normally, robbing bees originate from rather strong colonies while the invaded colony typically is characterized by ineffective guarding due to being diseased and weakened. If the robbed colony is suffering from AFB, a robber bee might bring *P. larvae* spores back to its own nest on the surface of its body or in robbed honey stored in its honey stomach. Supporting early notions on robbing as a route of *P. larvae* spore transmission between colonies (Hornitzky, 1998), a recent study convincingly demonstrated that robbing is a very efficient horizontal route of actual disease transmission between colonies and that this route is the more effective the higher the local colony densities are (Lindström et al., 2008b) suggesting that bee keeping practice facilitates this route of pathogen transmission.

Drifting bees are foragers which do not return to their own nest but accidentally fly into another colony. Although guard bees usually detect and repel bees from other colonies, a drifting bee might be able to bribe the guard bees by offering honey and eventually it will be allowed to enter the colony. The drifting of bees into the wrong colony occurs the more frequent the greater the colony density is (Jay, 1965; Goodwin et al., 1994; Pfeiffer and Crailsheim, 1998). While feral colonies are widely separated thereby precluding drifting of bees, bee keeping practice with many hives in a confined area is fostering this behaviour and, thereby, this route of pathogen transmission.

In addition to these 'natural' routes of horizontal transmission between colonies other routes artificially introduced by the bee keeper do exist. It is common bee keeping practice to exchange hive material like honey or brood combs between colonies, to reuse hive material when setting up a new colony, and to combine weak colonies to build a strong colony. If the used hive material is contaminated with *P. larvae* spores and/or the bee material is spore contaminated or even infected then

this will provide another horizontal route of AFB transmission again not occurring in feral colonies under natural conditions.

While the possibility of vertical transmission of *P. larvae* within colonies can be ruled out, vertical transmission of *P. larvae* spores or AFB between colonies is possible. Some colonies infected by *P. larvae* never develop clinical disease symptoms visible to the beekeeper; they survive and reproduce although infected (Hansen and Brodsgaard, 1999). Swarms budding off from such a diseased colony also may not develop disease symptoms (Hansen and Brodsgaard, 1999). These differences in disease progression were mainly attributed to differences in host tolerance (Woodrow and Holst, 1942; Sturtevant and Revell, 1953; Hoage and Rothenbuhler, 1966; Brodsgaard et al., 1998 and 2000; Spivak and Reuter, 2001) but are most likely also related to the described virulence differences between different *P. larvae* strains and genotypes (Genersch et al., 2005 and 2006).

A most recent study analysed the rate of vertical transmission of *P. larvae* between colonies in great detail (Fries et al., 2006). It was demonstrated that *P. larvae*-infected but not yet overtly clinically diseased colonies are able to reproduce by colony fission (swarming) as are clinically diseases colonies although with a much lower frequency. Spores were detectable in both, swarms and daughter colonies, directly after swarming. However, although spore density declined over time after swarming and no disease outbreak occurred during the observation time, the daughter colonies (i.e. the new queen in the old, contaminated nest with potentially diseased brood and part of the original bee colony) did not manage to totally eradicate the *P. larvae* spores. A year later, these colonies tested positive for *P. larvae* spores again, whereas the swarms (i.e. old queen accompanied by worker bees from the original colony set up on brand new hive material) became spore-free and remained so during the observation period. These results are a strong argument for the shook-swarm-method in conjunction with sterile hive material as a reliable means to cure AFB while saving at least the adult bees. Further long-term studies under normal bee keeping conditions are needed to verify these results and to enhance their impact for combating AFB in apiculture.

6. Diagnosis of AFB

AFB diagnosis is based on clinical symptoms and laboratory identification of *P. larvae*. Clinical symptoms at late stages of AFB include an obviously scattered brood nest with capped brood cells showing sunken, perforated cell caps. Larval remains forming a characteristic glue-like colloid (ropy thread) or dried-in foulbrood scales are highly specific clinical symptoms and considered reliable for the clinical diagnosis of AFB in the field. Considering the results on virulence differences between *P. larvae* genotypes (Genersch et al., 2005 and 2006) and their implications for the proportion of diseased larvae contributing to these clinical symptoms (Genersch, 2007) it is at least questionable whether or not a clinical diagnosis relying on the detection of ropy mass and scales is sufficient to detect diseased colonies in all circumstances. Infections caused by *P. larvae* ERIC II may be difficult to diagnose at early stages on the basis of the described symptoms (Tab. 1). In the evaluation of the health status of a colony, routine analysis of honey samples (Von der Ohe and Dustmann, 1997) or sampled adult bees (Lindström et al., 2008a) and genotyping of *P. larvae* (Genersch and Otten, 2003) – if present – may help to close this diagnostic gap.

Apart from these distinctive clinical symptoms, laboratory confirmation of *P. larvae* present in the suspect colony is required in most countries where AFB is a notifiable disease. For laboratory diagnosis, either vegetative *P. larvae* are directly cultivated from ropy larval remains or *P. larvae* spores are induced to germinate and then cultivated. For diagnostic purposes, spores can be isolated from various sources including honey ideally taken from brood combs (Shimanuki and Knox, 1988; Hornitzky and Clark, 1991; Von der Ohe and Dustmann, 1997), pollen (Gochnauer and Corner, 1974), wax (Gochnauer and Corner, 1974), winter hive debris (Titera and Haklova, 2003), and adult bees (Lindström and Fries, 2005). The latter source is reported to have the best predictive value for tracing risks for the development of clinical disease symptoms since a significant relationship between spore load of adult bees and mortality of honey bee larvae could be demonstrated experimentally (Lindström et al., 2008a).

Laboratory diagnosis of AFB is based on complex media allowing cultivation, germination, and sporulation of *P. larvae*. There are several diagnostic protocols for the detection of *P. larvae* spores in honey or bee samples, all including heat pretreatment of samples to eliminate contaminants and to stimulate germination of *P. larvae* (Dingman and Stahly, 1983; Hansen, 1984; Shimanuki and Knox, 1988; Hornitzky and Karlovskis, 1989; Steinkraus et al., 1998). A most recent analysis of the temperature dependent germination rate of different strains of *P. larvae* demonstrated clear differences between the ERIC-genotypes (Forsgren et al., 2008). According to the results presented germination of strains belonging to *P. larvae* ERIC I was highly stimulated by pre-treatment of the samples at temperatures of 90 °C and 95 °C whereas germination of representatives of genotype ERIC II was nearly abolished by the same procedure (Tab. 1). Therefore, the standard heat treatment in many protocols will favour germination and detection of *P. arvae* ERIC I and hamper the detection of *P. larvae* ERIC II. In epidemiological studies these differences in temperature sensitivity will distort the picture and lead to an overrepresentation of ERIC I strains compared to ERIC II strains (Genersch and Otten, 2003; Peters et al., 2006; Loncaric et al., 2008). In AFB diagnosis these differences will again cause problems in identifying ERIC II infected hives making *P. larvae* ERIC II a pathogen which is rather difficult to diagnose since clinical symptoms might become visible only at late stages of the disease and standard laboratory protocols for the detection of the pathogen in bee and honey samples might yield false negative results (Tab. 1). These new findings have to be addressed properly in future discussions about AFB diagnostics.

Once suspect colonies have grown on nutrient agar additional tests are necessary for *P. larvae* identification. Prior to the development of molecular tools, formation of giant whips upon sporulation (Plagemann, 1985), the absence catalase

activity (Ritter, 1996), and a characteristic biochemical profile (Gordon et al., 1973; Carpana et al., 1995; Dobbelaere et al., 2001b; Kilwinski et al., 2004) were commonly used to identify the pathogen. The recent reclassification of the species *P. larvae* (Genersch et al., 2006) finally simplified AFB diagnosis in the laboratory by opening the possibility to use already described PCR protocols (Govan et al., 1999; Dobbelaere et al., 2001a; Alippi et al., 2004; Neuendorf et al., 2004; De Graaf et al., 2006; Genersch et al., 2006) for the fast and specific identification of *P. larvae*.

7. Outlook

Now we are looking back on more than one hundred years of foulbrood research. Many problems could be solved and many questions could be answered but in doing so new problems and questions arose. Molecular methods have been finally introduced into this field of research and with the availability of the genomic sequence of *P. larvae* (Qin et al., 2006) and methods to manipulate this bacterium (Murray and Aronstein, 2008) we can start to analyse the interaction between the pathogen *P. larvae* and its host, the honeybee larvae, at the molecular level to better understand the biology of *P. larvae* and the pathology and pathogenesis of AFB.

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