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American foulbrood of the honey bee: Occurrence and distribution of different genotypes of *Paenibacillus larvae* in the administrative district of Arnsberg (North Rhine-Westphalia)

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With 2 figures and 1 table

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Summary

Between March 2003 and October 2004, *Paenibacillus larvae*, the aetiological agent of American foulbrood disease of the honey bee, was isolated from broodcombs and honey samples of 54 apiaries in the administrative district of Arnsberg (North Rhine-Westphalia, Germany). Genotyping of 176 *P. larvae* isolates with repetitive element polymerase chain reaction fingerprinting (rep-PCR) using BOX A1R and MBO REP1 primers revealed five different genotypes (AB, Ab, ab, aß, Aƀ). In samples of three apiaries, more than one genotype was detected. A combination of two genotypes was isolated from honey samples of the same hive two times (ab/aß and Ab/ab). The five genotypes were not randomly distributed in the district, but revealed a certain geographical clustering. Possible factors with impact on the genotype diversity and the distribution pattern are discussed.

Introduction

American foulbrood (AFB) is the most serious disease of the larval stage of the honey bee (*Apis mellifera* L.). It is caused by the Gram-positive, spore-forming bacterium *Paenibacillus larvae*. A recent polyphasic taxonomic study (Genersch et al., 2006) comprising morphological and biochemical tests, genomic fingerprints and exposure bioassays showed that former differentiation into two subspecies, *P. larvae* ssp. *larvae* and *P. larvae* ssp. *pulvifaciens* (Heyndrickx et al., 1996), is not justified and both are only variants of the species, *P. larvae*, without subspecies differentiation. Infection with *P. larvae* spores leads to decomposition of affected larvae and results in the collapse of the bee colony in most cases. The disease is highly contagious and infection spreads via transmission of resistant spores by bees within and between apiaries. This is often facilitated by common beekeeping practices like moving hives and exchange of combs between colonies (Hansen and Brødsgaard, 1999).

American foulbrood is notifiable in the Federal Republic of Germany. Official ascertainment of the disease by veterinary authorities is followed by drastic sequels like stamping out of infected colonies or initiation of artificial swarms, investigation of adjacent apiaries and introduction of quarantined areas around affected colonies with termination of any migration. Despite this rigorous policy, AFB is widely distributed in Germany. Moreover, there are vast regional differences in the number of AFB outbreaks. Observations over a 12-year period (1992–2003) revealed 0.8 outbreaks per 1000 colonies in North Rhine-Westphalia. In contrast, in the federal states of Bavaria and Baden-Wurttemberg, only 0.2 outbreaks per 1000 colonies were recorded, although these states have a higher density of bee colonies when compared with North Rhine-Westphalia (Otten and Otto, 2004). Climatic influences, differences in the prevalence of migratory beekeeping and different strains of *P. larvae* were suspected as possible reasons for these regional distinctions.

The last aspect was substantiated by the results of a genotyping study with *P. larvae* field isolates from various regions in Germany (Genersch and Otten, 2003) from which a geographic clustering of different genotypes became evident. Using repetitive-element polymerase chain reaction fingerprinting (rep-PCR), the authors examined 105 *P. larvae* isolates and detected four genetic subgroups (AB, Ab, ab, αB). The genotypes seemed not to be randomly distributed but revealed a geographical clustering. The few *P. larvae* isolates originating from North Rhine-Westphalia unexceptionally belonged to the same genotype (AB), which generally predominates in the western federal states of Germany.

Subsequently, genotyping of *P. larvae* with rep-PCR proved suitable as a molecular epidemiological tool to trace infection routes. Hence, autonomy of outbreaks of the disease was proven by demonstration of strains of different genotypes. Possible links between adjacent outbreaks became obvious by the isolation of strains of the same genotype (Genersch, 2003).

The high rate of AFB outbreaks in North Rhine-Westphalia and the lack of any information about the occurrence and distribution of *P. larvae* genotypes, was the rationale for this study.

Material and Methods

Broodcomb and honey samples infected with *P. larvae*

During 2003 and 2004, *P. larvae* was isolated from 32 broodcombs and 80 honey samples of 54 apiaries in the administrative district of Arnsberg, which is located in the south-east of North Rhine-Westphalia. It covers an area of 8250 km^2 and comprises 13 councils including six cities. A total of 176 isolates were collected from nine of 13 councils of the administrative district. Broodcombs were usually sent for bacteriological confirmation of clinical AFB, whereas honey

Table 1. *Paenibacillus larvae* isolates from 54 apiaries in the administrative district of Arnsberg according to the submitting council and sample matrix

Council	Number of infected apiaries	Number of *P. larvae* isolates	Infected broodcombs	Infected honey samples
BO	2	8	4	0
DO	7	22	7	4
UN	8	15	0	9
HAM	2	7	0	6
SO	1	3	0	3
MK	1	2	0	1
HSK	9	16	7	5
OE	12	53	11	21
SI	13	50	3	31
Total	54	176	32	80

BO, Bochum; DO, Dortmund; UN, Unna; HAM, Hamm; SO, Soest; MK, Märkischer Kreis; HSK, Hochsauerlandkreis; OE, Olpe; SI, Siegen.

samples were taken in the course of official investigations of bee colonies adjacent to a confirmed or suspected AFB colony or in the course of official investigations in order to lift quarantine. *P. larvae* isolates from honey samples were included in the study regardless of whether bee colonies were clinically diseased or asymptomatic. The number of infected apiaries, the number of *P. larvae* isolates and the number of broodcombs and honey samples infected with *P. larvae* are listed in Table 1. During the investigation, one to nine samples of 54 apiaries were positive for *P. larvae* resulting in one to 12 *P. larvae* isolates per beekeeper, respectively.

Bacteriological identification of *P. larvae*

For culture of *P. larvae* from broodcombs, decaying larval material was transferred onto Columbia agar supplemented with 5% sheep blood. Sheep blood agar plates were incubated for 3 days at 37 °C under microaerophilic conditions. Growing colonies were checked with 3% (v/v) H_2O_2 for catalase activity. Catalase-negative colonies were analysed by Plagemann test for formation of giant whips in the liquid part of Columbia sheep blood agar slants (Plagemann, 1985). Columbia sheep blood agar slants in screw cap plastic tubes were incubated at 37 °C for 6 days before preparations of the liquid at the bottom of the tubes were microscopically investigated for giant whips. Catalase-negative and giant whip-positive reactions were regarded as indicative of *P. larvae* and further verified by specific PCR detection. Honey samples were examined according to Von der Ohe and Dustmann (1997). One to four presumptive *P. larvae* isolates per broodcomb or honey sample were collected for further examinations.

PCR identification of *P. larvae*

For preparation of *P. larvae* DNA for PCR analysis, an isolated colony was scraped off the agar plate and resuspended in 180 μl of lysis buffer T1 (Macherey-Nagel, Düren, Germany). Isolation of DNA was performed using spin columns (Nucleospin Tissue Kit, Macherey-Nagel) in 100 μl of elution buffer according to the manufacturer's instructions.

The oligonucleotide primers chosen were complementary to a region of the *P. larvae* 16SrRNA gene (Govan et al., 1999): the reverse primer differed from the described primer in the substitution of an adenine base by a thymine base (5′-TCTATCTCTAAACCGGTCAGAGG-3′), while the forward primer was identical to the described primer (5′-AA-GTCGAGCGGACCTTGTGTTTC-3′). The primers produce an amplicon of 973 bp. The PCR mixture contained 5 μl of 10x buffer (Qiagen, Hilden, Germany), 250 μM (each) deoxynucleoside triphosphate, 500 nM forward and reverse primers, 0.4 U of HotStar *Taq* Polymerase (Qiagen) and 2 μl of DNA template in a total of 50 μl. Each PCR mixture was subjected to 40 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s and extension at 72°C for 60 s, with an initial denaturation at 95°C for 15 min. PCR products were examined by using agarose (1.5%) gel electrophoresis and visualized by using ethidium bromide and UV light.

Genotyping of *P. larvae* by rep-PCR analysis

Rep-PCR analysis using primers BOX A1R (5′-CTACGG-CAAGGCGACGCTGACG-3′) and MBO REP1 (5′-CCGCCGTTGCCGCCGTTGCCGCCG-3′) suitable for genotyping of *P. larvae* isolates was carried out according to the protocol of Genersch and Otten (2003).

Mapping of *P. larvae* genotypes

Paenibacillus larvae-infected bee colonies were mapped using the TierSeuchenNachrichten (TSN) software (version 2.3, Friedrich Löffler Institute, Wusterhausen, Germany). Geo reference data of infected bee colonies were supplied by local veterinary authorities.

Results

Identification and molecular subtyping of *P. larvae*

All 176 bacterial isolates which were regarded as indicative of *P. larvae* based on their negative catalase activity and their ability to produce giant whips were finally confirmed using the species-specific PCR.

Using rep-PCR with BOX A1R and MBO REP1 primers, five different genotypes were detected among the 176 *P. larvae* strains. Figure 1 shows fingerprints of five *P. larvae* strains, each representing a different genotype. With BOX A1R-primers, two banding patterns were distinguishable differing in the presence or absence of a band around 700 bp (Fig. 1a). According to Genersch and Otten (2003), these fingerprints were named A and a, respectively. With MBO REP1-primers, four banding patterns could be differentiated (Fig. 1b). Presence of a band around 1.1 kbp accompanied with the absence of a band around 1.03 kbp indicated subgroup B, and the reverse case subgroup b. Lack of both bands in the otherwise typical *P. larvae* banding pattern was indicative of subgroup ß. One *P. larvae* isolate exhibited a banding pattern with bands around 1.1 and 1.03 kB, simultaneously. This banding pattern with MBO REP1-primers had not been reported before and was named Б. A mixture of two genotypes (e.g. AB and ab), which would result in the same fingerprint, was excluded by the use of bacterial cultures derived from single colonies for DNA preparation. DNA preparations of several cultures always revealed the same fingerprint. The two BOX A1R patterns were found in five combinations with the four MBO REP1 patterns resulting in five different genotypes

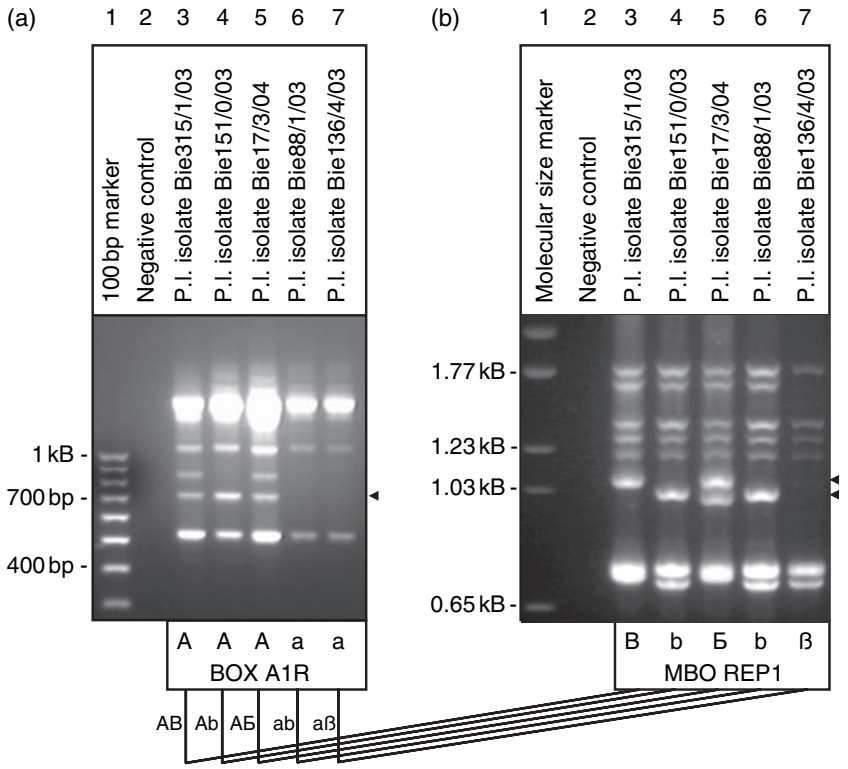

Fig. 1. rep-PCR fingerprint patterns of five representative *P. larvae* strains isolated in the administrative district of Arnsberg (North Rhine-Westphalia). (a) Using BOX A1R primers two different banding patterns corresponding subgroups A and a are visible among the five *P. larvae* strains (lanes 3–7). (b) Using MBO REP1 primers four different banding patterns corresponding subgroups B, b, Б and ß could be differentiated among the same strains (lanes 3–7). Taken together, each of the five *P. larvae* strains represents a different genotype detected in the administrative district of Arnsberg (AB, Ab, AБ, ab and aß).

of *P. larvae* (AB, Ab, AБ, ab, aß) in the administrative district of Arnsberg.

Genotypes AB and Ab were detected in samples of 21 apiaries (38.9%), respectively. *P. larvae* strains of genotype ab were isolated from samples of 14 apiaries (25.9%), and genotypes aß and AБ in samples of one apiary (1.9%), each. In three apiaries, a combination of different genotypes was detected. Two of them were infected with genotypes Ab/ab, and ab/aß, respectively. In one apiary, a combination of three different genotypes (AB/ab/AБ) was detected. Furthermore, in two instances, a combination of two genotypes was isolated from honey samples of the same hive (ab/aß and Ab/ab). Although all *P. larvae* isolates from broodcombs belonged to the same genotype this was not the case for *P. larvae* isolates from honey samples. All examined broodcombs from which isolates were selected showed clinical AFB with positive match stick test. From broodcombs with clinical symptoms of AFB, either genotypes AB, Ab or ab were isolated. These genotypes were also demonstrated in several honey samples. In contrast, all three *P. larvae* strains of genotype aß and the single *P. larvae* strain of genotype AБ originated from honey samples.

Distribution of *P. larvae* genotypes in the administrative district of Arnsberg

The distribution of the five different genotypes of *P. larvae* in the administrative district of Arnsberg within the 2-year investigation period is shown in Fig. 2. Apiaries from which *P. larvae* was isolated are marked with different symbols corresponding to the genotype detected. For reasons of clarity and because in a few cases more than one genotype was isolated from the same apiary, results are shown in four separate political maps.

Although genotype AB was detected only in northern (BO, DO, UN, HAM, SO) and southern councils (SI) of the district (Fig. 2a), genotype Ab occurred in the mid-zone (MK, HSK) and southern councils (OE, SI) (Fig. 2b). It was found to be the exclusive genotype within two central councils (MK, HSK). Occurrence of genotype ab was somewhat overlapping in the north (BO, UN, HAM) with that of genotype AB and in the south (OE, SI) with that of genotype Ab, but was not demonstrated within the mid-zone of the district (Fig. 2c). Genotypes aß and AБ were detected in two adjacent apiaries in the most northern council (HAM) (Fig. 2d).

Discussion

Paenibacillus larvae is not ubiquitous in Germany (Von der Ohe and Dustmann, 1997). Given this background, genotyping of *P. larvae* strains is a potent molecular epidemiological tool that can help to analyse associations between AFB outbreaks (Genersch, 2003). Eventually, even infection routes can be traced.

Despite this practical approach, information about distribution and occurrence of different genotypes of *P. larvae* worldwide (Alippi and Aguilar, 1998; Genersch et al., 2005) and in Germany (Genersch and Otten, 2003) is very limited so far. Staatliches Veteruäruntersuchungsamt Arnsberg is integrated in the official confirmation of AFB outbreaks and consecutive investigations in the administrative district of Arnsberg (North Rhine-Westphalia). Samples of nearly every AFB outbreak in the district are investigated. This gave rise to an epidemiological study on the occurrence and distribution of different *P. larvae* genotypes in the administrative district, in which around 2700 beekeepers with nearly 17 600 hives were officially registered in 2004.

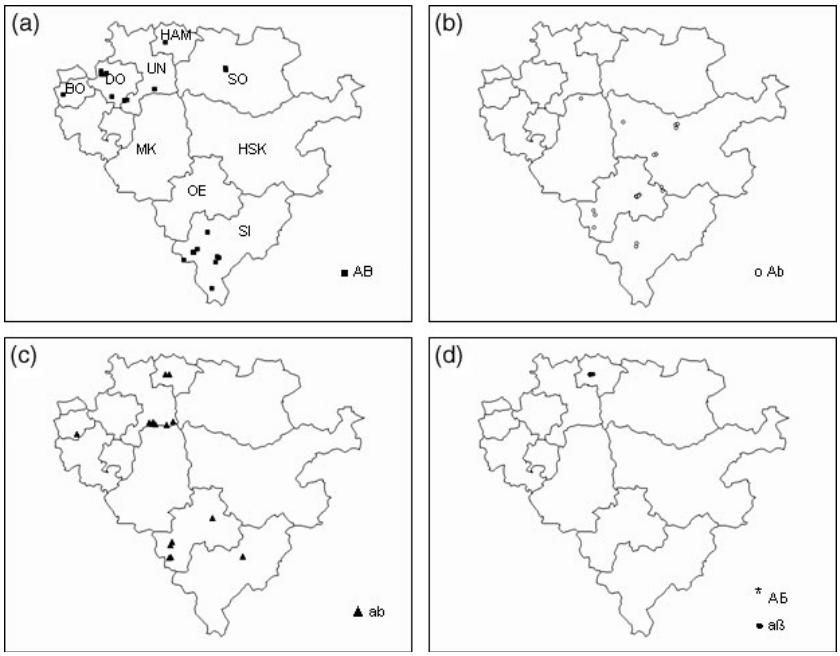

Fig. 2. Distribution of five genotypes of *P. l. larvae* (AB, Ab, ab, AБ, aß) in the administrative district of Arnsberg. Depicted are the locations of the bee colonies with detection of genotype AB (a), Ab (b), ab (c), and AБ, aß (d). Abbreviations of the councils in (a) are explained in footnote of Table 1.

In our study, 176 *P. larvae* strains obtained from 54 apiaries were investigated. From the results of voluntary AFB monitoring programmes in Rhineland and Westphalia (North Rhine-Westphalia) in 2003 and 2004, it was estimated that $< 10\%$ of the apiaries were infected with spores of *P. larvae* (T. Klüner, personal communication). Accordingly, the 54 apiaries of our study correspond with around one-fifth of the estimated infected apiaries in the administrative district. Investigation of the 176 *P. larvae* strains with rep-PCR using BOX A1R and MBO REP1 primers resulted in the detection of five different *P. larvae* genotypes. This is in contrast to the results of a previous rep-PCR study on *P. larvae* isolates in Germany (Genersch and Otten, 2003) in which all nine *P. larvae* isolates from North Rhine-Westphalia belonged to the same genotype (AB). Considering our results, the epidemiological AFB situation with regard to genetic strain diversity in North Rhine-Westphalia is much more complex. According to Genersch and Otten (2003), genotype AB prevailed in mid-western Germany. Not only are all investigated *P. larvae* isolates from North Rhine-Westphalia but also all strains from the adjoining federal state Lower Saxony and all but one *P. larvae* strain from Rhineland Palatinate belonged to this genotype. Because of the low numbers of investigated *P. larvae* strains per federal state, this finding has to be interpreted very carefully. Nevertheless, we could confirm that besides genotype Ab, genotype AB was the most frequently detected genotype in our region.

From four *P. larvae* genotypes (AB, Ab, ab, αB) detected in various regions of Germany (Genersch and Otten, 2003), three genotypes (AB, Ab, ab) could also be demonstrated in our relatively small district. In addition, we detected two further genotypes, aß and AБ. Isolates of genotype aß had been previously demonstrated in Baden Wurttemberg and Thuringia (E. Genersch, unpublished observation). Furthermore, the *P. larvae* reference strain (ATCC 9545) belongs to this genotype (Genersch et al., 2005). Genotype AБ was hitherto not reported. In our study, it was detected only in one apiary. The epidemiological situation gets even more complicated, because for the first time up to three *P. larvae* genotypes were detected within samples of the same apiary. In two instances, even two different *P. larvae* genotypes (ab/aß, and Ab/ab) were found in a honey sample of a single bee colony. Apiaries infected with more than one genotype were checked for predisposing factors. The beekeepers stated unanimously, that they had not migrated with their colonies, had not bought bees or bought used beekeeping equipment, and had not fed honey. On the other hand, exchange of combs between colonies of the same apiary was common practice. Only in one apiary with multiple *P. larvae* genotypes could a likely infection route be traced. A honey sample with two *P. larvae* genotypes (ab and aß) originated from a swarm of unknown origin. The *P. larvae* genotype ab had been detected in honey samples of the same apiary a year before. Therefore, it is conceivable that the swarm which was settled into an used but empty magazine introduced genotype aß into this apiary which was already infected with genotype ab.

Interestingly, clinical AFB at the colony level was always correlated with detection of a single genotype. Isolates from broodcombs with clinical symptoms of AFB (positive match stick test) of the same colony always belonged to the same genotype (AB, Ab and ab), whereas in two honey samples with a low spore contamination (Von der Ohe and Dustmann, 1997) two different genotypes were detected. Although genotype aß was not detected in association with clinical AFB in our sample material, it is known to produce clinical AFB symptoms in the field (E. Genersch, unpublished observations) and in an experimental exposure bioassay (Genersch et al., 2005). So far, genotype AБ had only been isolated once in this study from a honey sample of a colony without clinical disease. Therefore, nothing is known concerning its capability to produce clinical AFB. Coexistence of different genotypes in the same bee colony implicates the question of synergistic or antagonistic effects in the development of clinical AFB.

Distribution of *P. larvae* genotypes in the administrative district of Arnsberg

The distribution of the five *P. larvae* genotypes in our study showed a geographical clustering (Fig. 2a–d). Whereas in some regions there was overlapping in the occurrence of different genotypes, other regions were clearly dominated by specific genotypes. Geographic clustering of genotypes had also been observed by Genersch and Otten (2003) among isolates from different regions in Germany. Although our study disproves the genetic fingerprint homogeneity of *P. larvae* strains from North Rhine-Westphalia (Genersch and Otten, 2003), it indicates that certain genotypes may be enzootic in certain regions. As far as we can judge from the 2 years of investigation, the occurrence of predominating genotypes in councils which submitted samples in both years seemed to be relatively stable (data not shown).

Factors which might have an impact on the occurrence and the distribution pattern of *P. larvae* genotypes are the density of bee colonies and the migratory activity. The average number of bee colonies per square kilometre within a council seems to be positively correlated with the diversity of *P. larvae* genotypes in this council. Councils with detection of only a single genotype (HSK, MK, SO) often reveal a low density of bee colonies (HSK: 1.1–1.5 bee colonies/km^2, MK and SO: 1.6–2.0 bee colonies/km^2), whereas councils with three and more different genotypes (SI, UN, HAM) are characterized by high densities (> 3.0 bee colonies/km^2). Unfortunately, quality of data on the migratory activity of bee keeping in the councils differed largely. Therefore, only trends can be stated. Migration with bees was more common in northern councils with higher genotype diversity. Whereas there was a predominating efflux in the northern urban councils (Ruhrgebiet) during the bee season, other northern councils (SO, UN, HAM) were very attractive because of the common cultivation of rape and had therefore a considerable temporary migratory influx of bee colonies. Hence, also migratory activity might favour genetic diversity of *P. larvae* in certain regions.

Besides these external factors, variations in virulence of *P. larvae* strains and genotypes may have an influence on the spreading of the pathogen and therefore on the distribution pattern of genotypes in a defined area. Genersch et al. (2005a) demonstrated strain- and genotype-specific differences in virulence among *P. larvae* strains. In exposure bioassays, genotype AB killed infected larvae faster than other genotypes. As most larvae infected with genotype AB died before cell capping, it was hypothesized that nursing bees can remove them, thus diminishing the production of spores. Spreading of this genotype within the colony would therefore be more slow and clinical AFB diagnosis would be handicapped because fewer cells contain the ropy stage and foulbrood scale. Consequently, unrecognized spreading of genotype AB in the field by colony fission would be favoured. In contrast, AFB caused by one of the other *P. larvae* genotypes could be detected sooner because of obvious symptoms. Furthermore, spreading of genotypes other than AB would more often be disadvantaged by the official stamping out policy. In fact, besides genotype Ab, genotype AB was most frequently detected and had a wide regional distribution. Nevertheless, so far, a complete competitive displacement of other genotypes cannot be stated.

Continuation of this study in order to collect more data will be necessary to verify the distribution pattern and to analyse a possible shifting between genotypes. In conclusion, the administrative district of Arnsberg revealed a broad spectrum of different *P. larvae* genotypes occurring in a distinct geographic distribution pattern. If certain genotypes are truly enzootic in specific regions, genotyping will help veterinary authorities to trace back uncommon genotypes. Therefore, we recommend complementing routine bacteriological examination with genotyping of *P. larvae* isolates. Especially, bee colonies with detection of genotype AB in honey samples should be investigated very carefully for AFB symptoms.

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