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Rapid identification and genotyping of the honeybee pathogen *Paenibacillus larvae* by combining culturing and multiplex quantitative PCR

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Abstract
Background: American Foulbrood (AFB) is a devastating disease of honey bee (*Apis mellifera*) larvae caused by the spore-forming, Gram-positive bacterium *Paenibacillus larvae*. In most countries, the law requires mandatory reporting of AFB to the veterinary authority.
Aim and Methods: To speed up detection and genotyping of *P. larvae* spores, we compared different culturing protocols on Columbia sheep blood agar and developed a new multiplex quantitative polymerase chain reaction to distinguish between the two relevant *P. larvae* genotypes enterobacterial repetitive intergenic consensus (ERIC) I and ERIC II.
Results and Conclusion: As confirmed by *P. larvae* reference strains and field isolates, the new identification and genotyping protocol halves the time of current workflows, lessens labor-intension, allows a higher throughput of samples for monitoring, and permits a faster intervention to prevent the spread of AFB.
Keywords: American Foulbrood, ERIC genotyping, Multiplex quantitative PCR, *Paenibacillus larvae*, Rapid detection.

Introduction

Paenibacillus larvae, a Gram-positive, rod-shaped, flagellated and spore-forming bacterium, is the etiological agent of American Foulbrood (AFB), a deadly brood disease of honey bees (*Apis mellifera*) (Genersch, 2010). The infection starts with the oral uptake of *P. larvae* endospores by bee larvae below 36 hours of age (Yue *et al.*, 2008). The transmission of the extremely resilient and long-lived spores within or between bee colonies occurs by contaminated adult bees and spore-containing larval food. However, bee keepers contribute to the spread of AFB by feeding contaminated honey, moving diseased hives, or using contaminated equipment (von der Ohe *et al.*, 1996, 1997a, 1997b; Genersch, 2010). The *P. larvae* spores remain infectious for several decades, are resistant to heat and antibiotics, and about 10 are sufficient to infect and finally kill a larva (Woodrow and Holst, 1942; Genersch, 2010). After germination of spores, vegetative cells of *P. larvae* massively proliferate in the larval midgut and later invade the haemocoel. The bee larvae subsequently disintegrate into a ropy mass which dries down to a spore-containing, highly contagious scale. With the progress of the disease fewer bees reach adulthood and the population of the hive collapses (Yue *et al.*, 2008; Genersch, 2010; Fünfhaus *et al.*, 2013).
Until now five genotypes, *P. larvae* enterobacterial repetitive intergenic consensus (ERIC) I–V, have been identified based on ERIC sequences (Genersch and Otten, 2003; Genersch *et al.*, 2006; Beims *et al*., 2020). While *P. larvae* I and II are frequently isolated from AFB-infected colonies, the genotypes ERIC III and IV have not been isolated in the recent years (Genersch, 2010; Ebeling *et al*., 2016), whereas genotype ERIC V was isolated in 2013 from a honey sample, without having information about the effected colonies (Beims *et al*., 2020). ERIC I and II isolates differ in endospore resistance, rate of sporulation, and the strategy and time they need to kill their hosts (Genersch and Otten, 2003; Genersch *et al.*, 2005, 2006; Poppinga *et al.*, 2012; Fünfhaus *et al.*, 2013; Beims *et al.*, 2015; Morrissey *et al.*, 2015). Therefore, it is crucial to discriminate the slow killing phenotype of ERIC I (12 days) and the fast killing phenotype of ERIC II (7 days) (Genersch, 2010). Moreover, genotyping is necessary to refine AFB epidemiology and outbreak management (Morrissey *et al.*, 2015). However, methods for genotyping like MLVA still exist (Descamps *et al*., 2016), but the main routine diagnostic is based on *P. larvae* detection without any epidemiological information. Our method allows to distinguish between the slow (ERIC I) and the fast killing (ERIC II-V) genotypes, which increases the diagnostic information.
The high contagiousness and the severity of AFB are the reasons why this most devastating bacterial infection of honeybees is subjected to registration in many countries. Since the current treatment options for bee colonies suffering from AFB are very limited, it is very important to timely identify outbreaks and to confirm the source and routes of disease transmission (von

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der Ohe *et al.*, 1997a, 1997b; Morrissey *et al.*, 2015). Traditionally, the glue-like larval remains that can be drawn out as a thread with a matchstick represent a first-line warning practice of beekeepers (De Graaf *et al.*, 2006). Further clinical symptoms of AFB are diverse and depend on the genotype, the stage of disease, and the strength of the bee colony. Visible signs may include a patchy brood pattern, concave and perforated cell cappings, and flat scales tightly attached to the lower brood cell wall. Moreover, severely infected colonies emit a rotten odor (De Graaf *et al.*, 2006). However, obvious symptoms often need up to 3 years to develop and similar clinical signs can result from other bee diseases (von der Ohe *et al.*, 1996, 1997a, 1997b). According to this pathogenic development, colonies can be separated into uninfected ones, which do not harbor spores of *P. larvae*, and infected ones. These infected colonies might not show any visual symptoms in the first stages of infection, whereas spores of *P. larvae* can be detected in different matrices of the hive, i.e., food and comb material. Not till then, when *P. larvae* spread among the colony and a critical number of spores is reached, typical symptoms of AFB can be detected. In the past decades, considerable progress has been made in the taxonomic reclassification of *P. larvae* and in the diagnosis of AFB (Ash *et al.*, 1991; Govan *et al.*, 1999; Genersch and Otten, 2003; Genersch *et al.*, 2006; Morrissey *et al.*, 2015; Ruiz-Villalba *et al.*, 2017). Moreover, several sensitive and selective culture media and molecular techniques have been developed (De Graaf *et al.*, 2006; Amtliche Methodensammlung, 2017; Rossi *et al.*, 2018). Since cultural methods need confirmation by isolate identification, numbers of 16S rRNA-based polymerase chain reaction (PCR) protocols were established (Govan *et al.*, 1999; Dobbelaere *et al.*, 2001; OIE, 2008). However, these molecularbiological methods can only be used for the detection of vegetative *P. larvae* from larval remains or bacterial cultures. To specify these PCR-based protcols, we developed a multiplex quantitative PCR (mqPCR). This protocol allows reliable species identification by targeting 16S rRNA and ItuC gene sequences (Djukic *et al.*, 2014; Sood *et al.*, 2014). The ERIC genotyping is achieved by targeting gene sequences encoding for Plx1, an AB toxin which is present in ERIC I but not in ERIC II (Fünfhaus *et al.*, 2013).

Material and Methods

Bacterial strains and culture conditions

All of the bacterial strains used in this study are listed in Table 1. Bacterial reference strains were *P. larvae* DSM 7030 (ERIC I), DSM 25430 (ERIC II), DSM 17237 (ERIC II; RSK 16 standard) and *P. alvei* (DSM 29). Field isolates of *P. larvae* genotype ERIC I and II were originally derived from honey samples by the Lower Saxony State Office for Consumer Protection and Fodd Sefety, Institute of Apiculture, Celle, Germany (Beims *et al.*, 2015). If not stated otherwise, all strains

Table 1. Resulting amplifications by verifying and genotyping *P. larvae* strains by multiplexed qPCR with different targets by their "final call (ΔRn)". –, no amplification of the taget; +, amplification of the target.

***P. larvae* strain**	**16S rRNA**	**ItuC**	**plx1.3**
Paenibacillus larvae ERIC I			
DSM 7030	+	+	+
Isolate 5	+	+	+
Isolate 9	+	+	+
Isolate 11	+	+	+
Isolate 15	+	+	+
Isolate 22	+	+	+
Isolate 24	+	+	+
Isolate 25	+	+	+
Isolate 26	+	+	+
Isolate 29	+	+	+
Isolate 145	+	+	+
Isolate 146	+	+	+
Isolate 148	+	+	+
Isolate 153	+	+	+
Isolate 155	+	+	+
Isolate 157	+	+	+
Isolate 159	+	+	+
Isolate 160	+	+	+
Isolate 162	+	+	+
Isolate 174	+	+	+
Paenibacillus larvae ERIC II			
DSM 16116	+	+	–
DSM 25430	+	+	–
DSM 17237 (RSK16)	+	+	–
Isolate 1	+	+	–
Isolate 3	+	+	–
Isolate 6	+	+	–
Isolate 7	+	+	–
Isolate 17	+	+	–
Isolate 20	+	+	–
Isolate 23	+	+	–
Isolate 27	+	+	–
Isolate 28	+	+	–
Isolate 135	+	+	–
Isolate 137	+	+	–
Isolate 144	+	+	–
Isolate 146	+	+	–
Isolate 152	+	+	–
Isolate 156	+	+	–

(Continued)

P. larvae strain	16S rRNA	ItuC	plx1.3
Isolate 158	+	+	–
Isolate 161	+	+	–
Isolate 168	+	+	–
Paenibacillus alvei (DSM 29)	–	–	–
No Template Control (NTC)	–	–	–

were cultivated on house-made Columbia sheep blood agar (CSA-LAVES) at 37°C (https://www.dsmz.de/). Alternatively, purchased CSA agar plates from Oxoid or Becton Dickinson (BD) were used.

Germination of P. larvae spores on different preparations of CSA

Germination assays were performed with spore suspensions from *P. larvae* DSM 17237 (RSK16 standard; LAVES IB CE). Aliquots of 200 µl RSK 16 standard were spread on CSA-LAVES (homemade) and commercially available CSA-Oxoid (Oxoid) and CSA-BD plates BD plates. Germination rates of spores were quantified and compared after 3 and 6 days. The mean and its standard error were determined. Significance was tested by Student's *t*-test.

Multiplex quantitative PCR (mqPCR) for P. larvae

Material from *P. larvae* colonies was homogenized in 50 µl sterile, deionized water, and incubated at 95°C for 15 minutes and 250 rpm. To sediment the bacterial debris, the mixture was centrifuged for 5 minutes at 5,000 × *g*. The supernatant was then used as template for mqPCR. The sequences of primers and probes targeting *P. larvae* are shown in Table 2. Oligonucleotides were designed based on the 16S rRNA sequence of *P. larvae* and the ItuC and Plx1 encoding sequences (http://bioinfo.ut.ee/primer3-0.4./) using the genome data of *P. larvae* DSM 7030 and DSM 25430 (Fünfhaus *et al.*, 2013; Djukic *et al.*, 2014; Sood *et al.*, 2014; Hertlein *et al.*, 2016; Rossi *et al.*, 2018). The mqPCR reactions were performed in a total volume of 20 µl containing 10 µl 2× Luna Universal Probe qPCR Mix (NEB, Germany), 0.4 µM of each primer (eurofins genomics, Germany), 0.2 µM of each probe (eurofins genomics, Germany), 2.0 µl template and nuclease-free water to reach the reaction volume. The amplification was performed in an ARIA MX qPCR thermocycler (Agilent, USA), according to the manufacturer instruction (initial denaturation 95.0°C; 40 cycles: 95.0°C for 15 seconds and 60.0°C for 30 seconds, including detection of amplification at the end of the 60.0°C step). A no template control (NTC) was used, as well as *P. larvae* reference strains for genotype ERIC I and ERIC II (positive controls), and *P. alvei* as negative control. Amplification was analyzed using the *Aria Agilent 1.3* software (Agilent, USA). Targets were interpreted as positive ("detected") when *Finall Call* (ΔRn) was marked as "+" by the software, with a quantification cycle (*Cq*) (ΔRn) ≤ 30 (Ruiz-Villalba *et al.*, 2017).

Ethical approval

All experiments were performed according to actual ethical guidelines.

Results and Discussion

Traditionally, monitoring of AFB is based on the observation of clinical symptoms within the apiary and microbial cultivation of *P. larvae* from honey, food, and brood samples (De Graaf *et al.*, 2006). All cultivation protocols for the detection of sub-clinical levels of the pathogen include a heat treatment of the samples to reduce contamination and to stimulate the germination of *P. larvae* spores (Forsgren *et al.*, 2008). Moreover, Nalidixid Acid can be used to avoid the growth of Gram-negative bacteria (Amtliche Methodensammlung, 2017). In our process, we reduced Gram-negative contaminants by a sample-processing step at 95°C. However, differences in heat resistance and germination rate of spores, as well as variation in proliferation among different *P. larvae* genotypes and strains may lead to a biased diagnose and disease monitoring (Forsgren *et al.*, 2008). Not surprisingly, a

Table 2. Primer and Probes used for detection and genotyping of *P. larvae* by multiplexed qPCR.

Used oligonucleotide	Sequence [5′→ 3]
16S-rRNA_1	CTGCCTGTAAGACCGGGATA
16S-rRNA_2	CGGAAGATTCCCTACTGCTG
p16S-rRNA	[FAM]GGATAGCTGGTTTCTTCGCA[BHQ1]
plx1.3_1	TAGCAGCAGCAATTCAGACG
plx1.3_2	GTGGCGATCCAACTTCACTT
pplx1.3	[CY5]AACTCAAGACATGGCAGCAACTCT[BHQ2]
ItuC_1	GCAGTGTTCCAAGCTTCTCC
ItuC_2	CGTATGGGAGCTGTTTTGGT
pItuC	[HEX]CACGATATGCTTCGGATCCT[BHQ1]

FAM = 6-FAM-phosphoramidit; CY5 = Cyanine5; HEX = Hexachloro-Fluorosceine; BHQ = Black hole Quencher [Biosearch Technologies Inc.].

high variability of bacterial plate counts from infected colonies has been reported (von der Ohe *et al.*, 1997a, 1997b). Since selection of the growth media may also influence quantification of the pathogen (Nordström and Fries, 1995), we compared the germination of *P. larvae* spores on CSA, because of its ready-to-use disposability, instead of other media like MYPGP. Three different CSA without Nalidixid Acid were tested for its sensitivity against *P. larvae*. Therefore, we used freshly prepared CSA-LAVES and purchased CSA-Oxoid and CSA-BD plates, respectively (Fig. 1). The average germination rate of *P. larvae* DSM 17237 spores (RSK 16 standard) revealed moderate differences on CSA-LAVES (n = 123) and CSA-Oxoid (n = 120) after 3 and 6 days, respectively. After 3 days of incubation germination on CSA-LAVES (10.1 ±0.33 cfu/plate) and CSA-Oxoid (7.39 ± 0.27 cfu/plate) plates differed by factor 0.7 ($p = 1.44 \times 10^{-9}$). In contrast, the bacterial plate counts on CSA-BD (n = 120) were much higher at both time points. Germination on CSA-LAVES and CSA-BD (76.32 ± 0.68 cfu/plate) plates differed by factor 7.6 ($p < 2.2 \times 10^{-16}$) (Fig. 1). After 6 days of incubation CSA-LAVES (21.49 ±0.44 cfu/plate) and CSA-Oxid (19.19 ± 0.41 cfu/plate) differed by factor 0.9 ($p = 1.71 \times 10^{-4}$), whereas CSA-LAVES and CSA-BD (86.61 ± 1.05 cfu/plate) differed by factor 4 ($p < 2.2 \times 10^{-16}$) (Fig. 1). The moderate differences between CSA-LAVES and CSA-Oxoid may be explained with the fresh sheepblood used for CSA-LAVES or the age of the purchased agar plates. However, the efficient germination and growth rate on CSA-BD suggests that additional ingredients of this complex medium are responsible for the marked difference. This is supported by our observation that cfu values of *P. larvae* on CSA-LAVES and CSA-Oxoid media are increasing over a time period of 6 days, while significantly higher cfu values on CSA-BD are already present after 3 days of cultivation. Thus, the faster and higher germination and growth rate after only 3 days suggests CSA-BD for a time-optimized protocol.

To timely verify culture-based identification of *P. larvae* strains and to differentiate between the epidemiologically prevalent *P. larvae* genotypes ERIC I and II, we developed and optimized a multiplex qPCR. With this protocol *P. larvae* reference strains (DSM 7030 ERIC I, DSM 25430 ERIC II) and 40 field isolates could be confirmed based on the amplification of the 16S rRNA target sequence (*Cq-value* ≤ 30) (Table 1), while no amplicons were detected for *P. alvei* (no *Cq*) and the NTC (*Cq-value* > 30). Similarly, PCR products of the ItuC target gene were detected for all *P. larvae* reference strains and field isolates (*Cq-value* ≤ 30). The NTC showed no *Cq* and *P. alvei* resulted in *Cq*-values greater or equal 35. Since *P. larvae* genotypes ERIC I and II differ in their virulence gene repertoir (Fünfhaus *et al*., 2013; Djukic *et al.*, 2014; Sood *et al.*, 2014; Ebeling *et al*., 2016), we utilized the ERIC I specific AB toxin Plx1 gene to discriminate between them in our mqPCR approach. As shown in Table 1, target sequence *plx1.3* was only detected in *P. larvae* DSM 7030 (*Cq* ≤ 30) and the field isolates of genotype ERIC I. *Paenibacillus larvae* DSM 25430 and all field isolate of genotype ERIC II were negative for *plx1.3*, whereas the *Cq-values* of the NTC and *P. alvei* were greater or equal 35. Thus, together with the results for the 16S rRNA and ItuC target sequences *P. alvei* can clearly be excluded and ERIC genotypes I and II of *P. larvae* be distinguished.

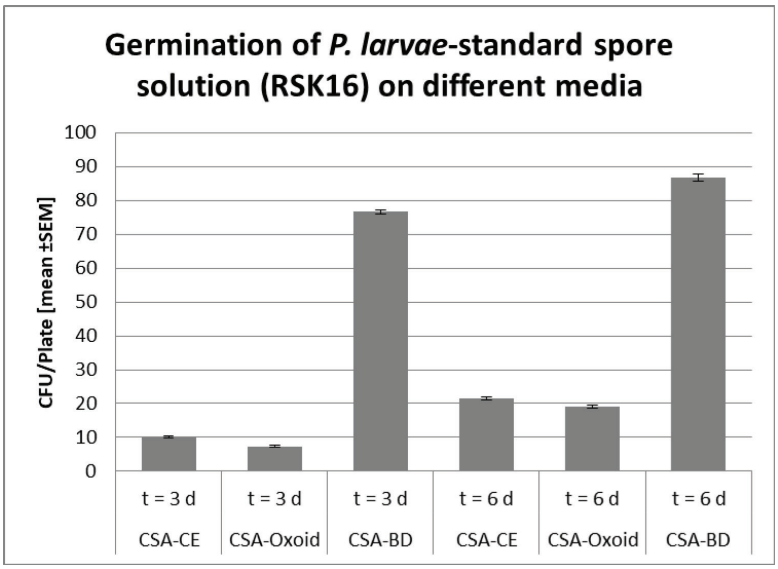

Fig. 1. Germination of *P. larvae*-stanadard spore solution (RSK16) on different Columbia Sheepblood-Agar. Significance was calculated by Student's *t*-test (***, $p < 0.001$).

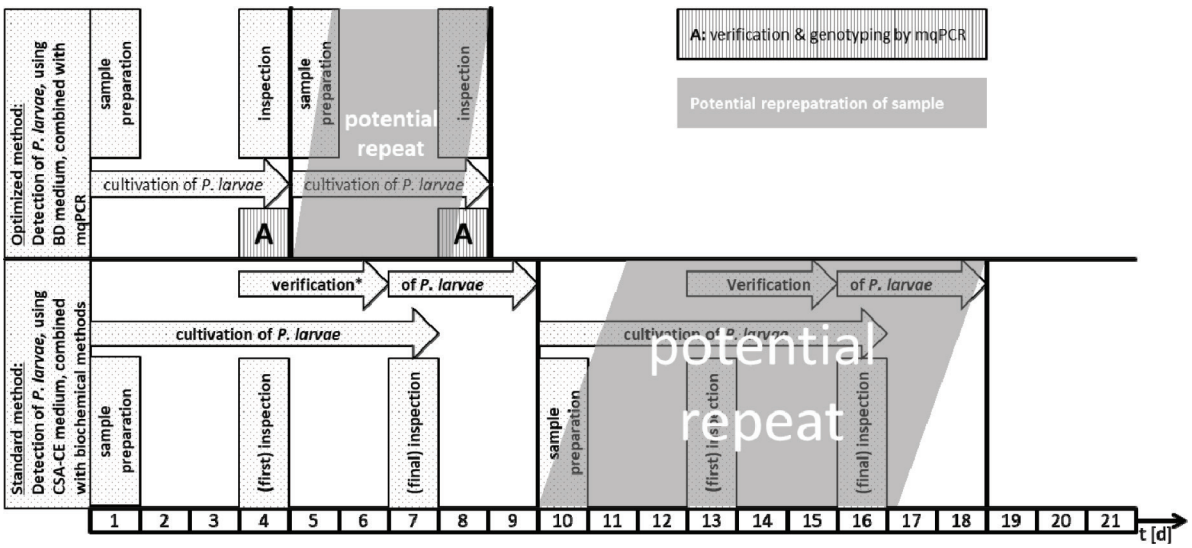

Fig. 2. Schematic comparison of the current standard culture-based method to detect *P. larvae* in honey and food samples and the newly developed protocol which combines optimized culture and mqPCR.

Conclusion

By combining a culture-based approach on CSA-BD with multiplex qPCR, we were able to detect and genotype *P. larvae* reference strains and field isolates within 4 days (Fig. 2). This is half of the time needed with the actual official standard protocol (Amtliche Methodensammlung, 2017). Since time consuming methods, such as purification of bacterial DNA, gel electrophoresis, and interpretation of fragment patterns are not necessary, the newly developed multiplex qPCR allows a higher throughput of samples, may reduce costs for monitoring surveys, and permit faster interventions to prevent the spread of AFB.

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Author's contributions

Hannes Beims designed the experiments for mqPCR and was involved in data analysis of germination assays. Martina Janke and Werner von der Ohe provided the germination assays and verified analytical methods. Michael Steinert analyzed the derived data. All authors discussed the results and contributed to the final manuscript.

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