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ORIGINAL ARTICLE

A real-time PCR-based strategy for the detection of *Paenibacillus larvae* vegetative cells and spores to improve the diagnosis and the screening of American foulbrood

J. Martínez[1], V. Simon[1], B. Gonzalez[2,3] and P. Conget[1]

1 Instituto de Ciencias, Facultad de Medicina, Clinica Alemana Universidad del Desarrollo, Santiago, Chile
2 Facultad de Ingenieria y Ciencias, Universidad Adolfo Ibañez, Santiago, Chile
3 Center for Advanced Studies in Ecology and Biodiversity, P. Universidad Catolica de Chile, Santiago, Chile

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Correspondence
Paulette Conget, Instituto de Ciencias Facultad de Medicina Clinica Alemana Universidad del Desarrollo, Av Las Condes 12438, Lo Barnechea, Santiago 7710162, Chile. E-mail: pconget@udd.cl

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Abstract

Aim: To develop a real-time PCR-based strategy for the detection of *Paenibacillus larvae* vegetative cells and spores to improve the diagnosis and the screening of American foulbrood (AFB), the most harmful pathology of honeybee brood.

Methods and Results: A real-time PCR that allowed selective identification and quantification of *P. larvae* 16S rRNA sequence was developed. Using standard samples quantified by flow cytometry, detection limits of 37·5 vegetative cells ml^{-1} and 10 spores ml^{-1} were determined. Compared to spread plate method, this real-time PCR-based strategy allowed, in only 2 h, the detection of *P. larvae* in contaminated honeys. No false-positive results were obtained. Moreover, its detection limit was 100 times lower than that of the culture method (2 *vs* 200 spores g^{-1} of honey).

Conclusion: A rapid, selective, with low detection limit, sensitive and specific method to detect and quantify vegetative cells and spores of *P. larvae* is now available.

Significance and Impact of Study: In addition to honey samples, this real-time PCR-based strategy may be also applied to confirm AFB diagnosis in honeybee brood and to screen other apiary supplies and products (bees, pollen, wax), thus broadening the control of AFB spreading.

Introduction

American foulbrood (AFB) causes significant economic losses to beekeepers, because it is the most harmful pathology of honeybee brood. The causative agent of AFB is *Paenibacillus larvae* (Genersch *et al.* 2006), whose only host is the honeybee *Apis mellifera*. Spores of *P. larvae* are the main vectors for the spreading of the disease (Haseman 1961). In infected hives, they can be found in brood but also in honey, wax, pollen and hive walls (Bakonyi *et al.* 2003). *P. larvae* spores are transported among apiaries by drifting bees and also by supplies, mainly honey (Delaplane 1991). It has been demonstrated that spores remain infective in the field for more than 35 years (Haseman 1961).

Diagnosis of AFB is based on hives visual inspection (De Graaf *et al.* 2006). This procedure presents clear limitations because it depends on the judgment of an expert and relies on the observation of clinical symptoms that are not always easily recognized (Malcom 1987; Lindström *et al.* 2008). The confirmation of the visual AFB diagnosis requires culturing and subsequent morphological, biochemical and physiological characterization of bacterial isolates (Hendrick *et al.* 1996). Laboratory tests currently available are useful to confirm the presence of *P. larvae* in infected hives but do not allow epidemiological and surveillance studies. Molecular techniques for the identification of *P. larvae* have been already published (Alippi and Aguilar 1998; Govan *et al.* 1998; Yang and Yoon 2001; Alippi *et al.* 2002). Most of

them are based on the detection, by standard PCR methods, of *P. larvae* gene sequences in DNA samples isolated from bacterial colonies grown on semi-selective medium (Dobbelaere *et al.* 2001; Lauro *et al.* 2003). A PCR-based assay for the detection of *P. larvae* vegetative cells in DNA extracts from infected larvae is also available (Lauro *et al.* 2003; Sang-Hoon *et al.* 2008). In the case of *P. larvae* spores, their detection and quantification have been performed by the spread plate method (Leuschner *et al.* 2003). For this, the antibiotic, pipemidic and nalidixic acids containing J medium, is used because it allows the growth of *P. larvae* and avoids the growth of other related bacterial species (Alippi 1995). This culture-based strategy is time consuming because it requires several days of incubation, it may overestimate the amount of spores because of the growth of false-positive colonies and does not allow the detection of spores that do not germinate *in vitro*. The latter is the most significant limitation of culture-based methods because it has been shown that only 6% of total *P. larvae* spores germinate under culture conditions (Dingman and Stahly 1983). To overcome this limitation, conventional PCR has been applied to detect *P. larvae* spores in honey (Bakonyi *et al.* 2003; D'Alessandro *et al.* 2007). These procedures have several pitfalls: (i) methods used to extract DNA from spores are lengthy and laborious, (ii) conventional PCR has restricted detection limit and, (iii) assay detection limit is determined against a sub-optimal spread plate method.

Our goal was to develop a rapid, selective, with low detection limit, sensitive, specific and quantitative assay to detect vegetative cells and/or spores of *P. larvae* in honeys. We set up a specific real-time PCR for *P. larvae* 16S rRNA sequence. Based on it, we established a strategy with experimentally contaminated honeys and proved its assay sensitivity and specificity against the spread plate method using field honey samples.

Materials and Methods

Culture and characterization of *P. larvae* JM87

P. larvae JM87 was an in-house isolate characterized microscopically and biochemically (Hornitzky and Nicholls 1993; De Graaf *et al.* 2006). Its culture was performed in J medium: 0·5% tryptone (Mo Bio Laboratories Inc., Solana Beach, CA), 0·3% K_2HPO_4 (Merck, Darmstadt, Germany), 1·5% yeast extract (Difco), 0·2% glucose, 20 μg ml^{-1} pipemidic acid (Sigma) and 9 μg ml^{-1} nalidixic acid (Sigma), pH 7·3–7·5 (Alippi 1995). When cultivation was carried out on solid medium, 2% agar was used. Cultures were maintained under microaerobic atmosphere (5% O_2, 5–10% CO_2) at 37°C.

For genetic identification of *P. larvae* JM87, DNA obtained from the isolate was amplified with the primers 8F (5′AGAGTTTGATCCTGGCTCAG3′) (Lane 1991) and 1392R (5′ACGGGCGGTGTGTAC3′) (Lane *et al.* 1985), targeted to bacterial 16S rRNA gene sequence. Briefly, reactions of 50 μl containing 50 ng of DNA, 0·2 μmol l^{-1} of each primer, 3 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} dNTP, 0·2 mg ml^{-1} BSA, PCR buffer (200 mmol l^{-1} Tris–HCl, pH 8, 500 mmol l^{-1} KCl) and 1 U of Taq polymerase were cycled as follows: one cycle 94°C, 5 min; 25 cycles 94°C, 45 s/56°C, 45 s/72°C, 2 min; one cycle 72°C, 7 min. The 1087-bp product was purified using the extraction kit Wizard®SV Gel and PCR Clean-Up System (Promega, Madison, WI). DNA sequencing was performed using the automated DNA sequencer ABI Prism 3100 (Applied Biosystems, Foster City, CA).

Culture of other bacterial species

Paenibacillus alvei III3 DT-1A, *Paenibacillus polymyxa* ATCC 842T, *Paenibacillus macerans* BKM B-51, *Bacillus licheniformis* ATCC 8480, *Bacillus subtilis* SB305, *Bacillus megaterium* ATCC 19213 and *Bacillus pumilus* ATCC 7061, obtained from the Bacillus Genetic Stock Center (Ohio State University, Columbus, OH) and *Escherichia coli* were maintained on Luria-Bertani Broth agar (Difco) and incubated under aerobic atmosphere at 37°C (Sambrook and Russel 2001).

DNA isolation

DNA isolation from *Paenibacillus* and *Bacillus* species was performed with the QIAamp® genomic DNA isolation mini kit for Gram-positive bacteria (Qiagen Inc., Valencia, CA). DNA was eluted with 200 μl of elution buffer.

DNA isolation from *P. larvae* spores was performed with the UltraClean Soil DNA isolation kit (Mo Bio Laboratories, Inc). DNA was eluted with 50 μl of elution buffer.

Bacterial DNAs were stored at −20°C until used as template for PCR amplification with primers 8F/1392R (internal control) and PL2-Fw/PL2-Rev.

Real-time PCR for *P. larvae* 16S rRNA sequence

Real-time PCR was performed in a final volume of 10 μl containing 1 μl of template DNA, 0·5 μmol l^{-1} primers PL2-Fw/PL2-Rev, 1·6 mmol l^{-1} $MgCl_2$ and 1 × SYBR Green Mastermix (Roche, Mannhein, Germany). PL2-Fw (5′CGGGAGACGCCAGGTTAG3′) and PL2-Rev (5′TTCTTCCTTGGCAACAGAGC3′) were designed based on the sequence of the *P. larvae* subsp. *larvae* 16S rRNA gene (GenBank accession number AY030079). Reactions with-

out template DNA were run as negative control. PCR amplification was carried out as follows: one cycle 95°C, 10 min; 40 cycles 95°C, 10 s/57°C, 10 s/72°C, 15 s; one cycle of melting temperature analysis (0·1°C s^{-1} up to 95°C). A LightCycler instrument (Roche) was used for amplification and data acquisition.

Amplicon melting temperature was determined using the LightCycler software version 3.5 (Roche). Its size was estimated by electrophoresis in 1·2% agarose gel stained with ethidium bromide (Sambrook and Russel 2001). The PCR product was ligated into a plasmid and cloned in *E. coli* using the pGEM-T cloning kit (Promega). Recombinant pJMPL-2 plasmid restriction analysis was performed with endonucleases *Eco*RI and *Sal*I.

P. larvae JM87 vegetative cells and spores stocks preparation

P. larvae JM87 was cultured in 20 ml of liquid medium on a rotary shaker (180 rev min^{-1}) at 37°C. At day 5, the culture was centrifuged at 9000 ***g*** for 3 min, washed and resuspended in one ml of sterile phosphate-buffered saline (PBS). For spores preparation, *P. larvae* JM87 was cultured in 10 ml of liquid medium in a closed conical tube without agitation, at 37°C. After 20 days, the culture was centrifuged at 9000 ***g*** for 5 min, washed and resuspended in one ml of PBS. Remaining vegetative cells were eliminated by filtration through sterile hydrophobic cotton. Spores were centrifuged at 9000 ***g*** for 5 min and resuspended in sterile distilled water to avoid germination (Laflamme *et al.* 2004). Stocks (10^6 vegetative cells or spores ml^{-1}) were kept at 4°C.

Epifluorescence microscopy

Vegetative cells or spores were stained with 0·5 μmol l^{-1} SytoBC (Bacteria Counting Kit, Molecular Probes Invitrogen, Carlsbad, CA) for 20 min at 37°C. After 5 min of staining, samples were mounted in glass slides and observed under a Laser Axioskop 2 epifluorescence microscope (488 nm, 505–550 BP filter) using a × 63/1·4 oil immersion plan-apochromat objective and ×10 oculars (Carl Zeiss, North Ryde, NSW, Australia).

Flow cytometry

Vegetative cells or spores diluted (10^{-6}) in sterile isotonic buffer were stained with 0·5 μmol l^{-1} SytoBC for 20 min at 37°C. Just prior to data acquisition, 5 μl of 1 mg ml^{-1} propidium iodide (Molecular Probes Invitrogen) prepared in PBS and filtrated through 0·2 μm was added to each sample. Also, a fixed number of 6-μm microspheres (Molecular Probes Invitrogen) were mixed with the stained cells. Samples were examined in a CyAn ADP flow cytometer (emission 488 nm, FL1 530/40 nm, 545 DLP and FL3 613/20 nm, 640 DLP) (Dako, Carpinteria, CA), and data were analysed with Summit 4.3 software (Dako). Forward scatter (FS) (voltage 400, amplifier gain 99·0), side scatter (voltage 425, amplifier gain 90·0) and fluorescence (FL1 voltage 534, amplifier gain 1·0 and FL3 voltage 690, amplifier gain 1·0) data were collected with logarithmic signal amplification. Mixtures of vegetative cells, spores and microspheres were used to frame three regions in FS *vs* fluorescence plots: vegetative cells-gate (R1), spores-gate (R2) and microspheres-gate (R3). The concentration of stocks was calculated as:

$$\text{concentration (vegetative cells ml}^{-1}) = [\text{number of events in R1} \times (\text{number of microspheres added/number of events in R3})] \times 10^6.$$

$$\text{concentration (spores ml)}^{-1} = [\text{number of events in R2} \times (\text{number of microspheres added / number of events in R3})] \times 10^6.$$

Preparation of experimentally contaminated honeys

Honeys from apiaries without clinical AFB symptoms were checked for the absence of *P. larvae* by culture and by real-time PCR. Fifteen grams of *P. larvae*-free honey was inoculated with one ml of *P. larvae* spore suspensions obtained by serial dilution in sterile water of spore stocks.

Quantification by spread plate and real-time PCR of *P. larvae* in honeys

Fifteen grams of experimentally contaminated honey or 27 g of field honey was vigorously mixed with 10 or 20 ml of sterile water, respectively, and centrifuged at 6000 ***g*** for 45 min. The pellet was washed, resuspended in one ml of sterile water, incubated at 80°C for 15 min and plated on three J medium plates. Viable spore counts were recorded by counting the colony-forming units (CFU) after 5 days of incubation at 37°C in microaerobic atmosphere. A colony glossy, discoloured, with irregular edges and a diameter of 1–3 mm that under the light microscope (×4) had a convoluted surface, was scored as a *P. larvae* CFU. Another ml of resuspended pellet was subjected to DNA isolation and real-time PCR. The absolute quantification was carried out using the fit points option and based on C_T values. Tenfold serial dilutions of experimentally contaminated

honeys (2–200 spores g^{-1} of honey) were used as standard curve. Field honeys *P. larvae* loads were calculated from standard curve plots (log input quantity *vs* C_T) (VanGuilder *et al.* 2008).

Results

Selective and quantitative real-time PCR detection of *P. larvae* 16S rRNA sequence

A similarity search using the Basic Local Alignment Search Tool (Altschul *et al.* 1990) showed 99% identity between *P. larvae* JM87 and *P. larvae* subsp. *larvae* sequences. DNA obtained from strain JM87 was used as template for real-time PCR with primers PL2-Fw/PL2-Rev. As expected, the PCR product melting temperature was 89·7°C (Fig. 1a), and its size was approximately 380 bp (Fig. 1b). Amplicon was cloned in pJMPL-2, whose *Sal*I and *Eco*RI digestion products were about 3400, and 3100 (vector) and 380 bp (amplicon), respectively (Fig. 1d).

Primers PL2-Fw/PL2-Rev selectivity was assessed using as template DNA isolated from *Paenibacillus* and *Bacillus* species that can be found in honey (Gilliam 1978). No amplification was observed when *P. alvei, P. polymyxa, P. macerans, B. licheniformis, B. subtilis, B. megaterium* or *B. pumilus* DNA samples were tested (Fig. 1b). The selectivity of primers PL2-Fw/PL2-Rev was further supported by the detection of the expected PCR product when using primers 8F/1392R (Fig. 1c).

The dynamic range of the real-time PCR was evidenced with tenfold serial dilutions of pJMPL-2 ($2–10^5$ copies per reaction). The technique detection limit was two copies of 16S rRNA sequences/reaction (data not shown).

Real-time PCR-based detection and quantification of vegetative cells and spores of *P. larvae*

The size of the cells as well as viability and purity of stocks was assessed by epifluorescence microscopy and flow cytometry. *P. larvae* JM87 vegetative cells measured 2–3 μm × 0·8–1·0 μm and spore diameter was 2 μm. Images evidenced neither cellular debris nor spore in vegetative cell stocks. In turn, spore stocks were free of debris and vegetative cells (data not shown). Flow cytometry data supported the absence of death cells (>95% excluded propidium iodide) and the homogeneity of *P. larvae* stocks (Fig. 2a,b, inserts). Spores were clearly differentiated from vegetative cells by their lower size (FS range 1–10 *vs* 10–100, respectively) and their lower nucleic acid content (FL1/SytoBC range 300–1000 *vs* 1000–10 000, respectively). Tenfold serial dilutions, quantified by flow cytometry, were subjected to real-time PCR for the detection of *P. larvae* 16S rRNA sequence. Peaks at the expected melting temperature were observed in samples containing as low as 37·5 vegetative cells ml^{-1} (Fig. 2a) or 10 spores ml^{-1} (Fig. 2b).

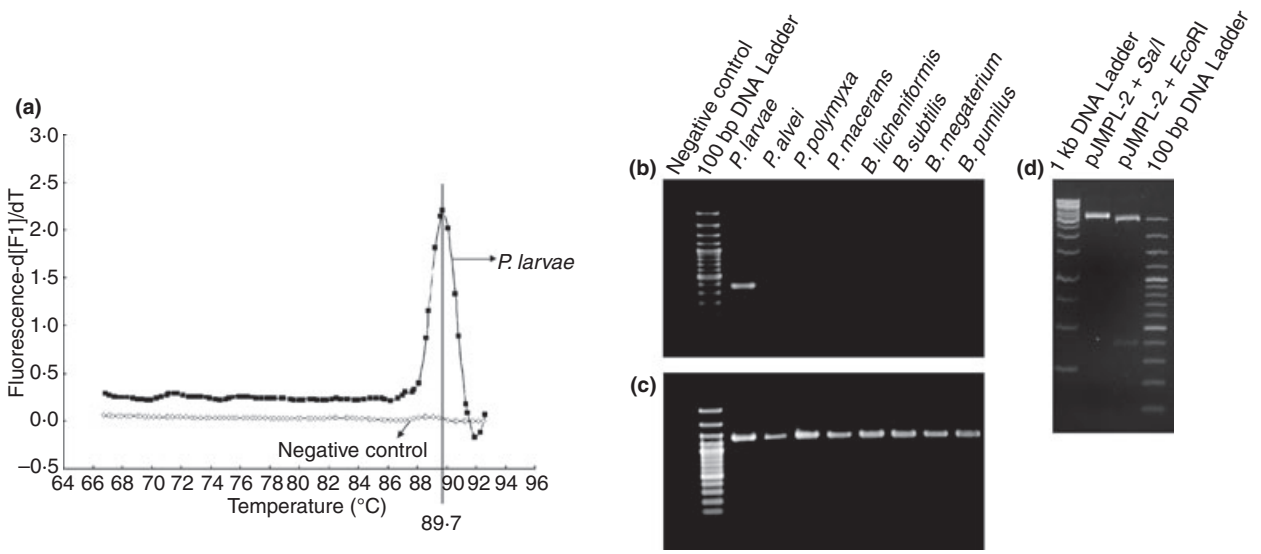

Figure 1 Real-time PCR for 16S rRNA sequence of *Paenibacillus larvae*. Melting curve of the PCR product obtained when *P. larvae* JM87 DNA was used as template and PL2-Fw/PL2-Rev, as primers (a). Agarose gel electrophoresis of the PCR products obtained when DNA of *P. larvae* JM87 and related bacteria was used as template and PL2-Fw/PL2-Rev (b) or 8F/1392R (c), as primers. Agarose gel electrophoresis of pJMPL-2 DNA plasmid after restriction endonuclease analysis (d).

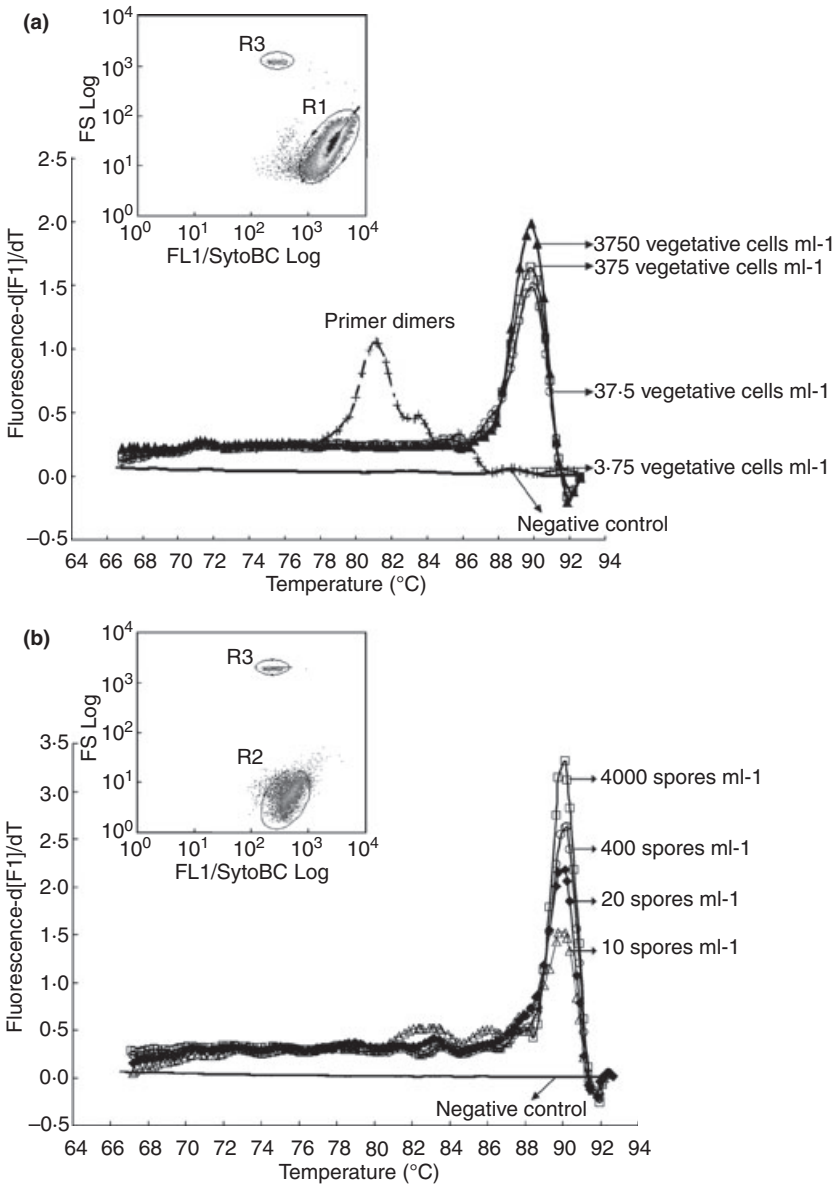

Figure 2 Detection and quantification by real-time PCR of vegetative cells and spores of *Paenibacillus larvae*. Melting curve of the PCR product obtained when DNA isolated from serial dilutions of vegetative cell (a) or spores (b) of *P. larvae* were used as template and PL2-Fw/PL2-Rev, as primers. Inserts show flow cytometry analysis of vegetative cells (a) and spores (b) stocks stained with SytoBC. R1, R2 and R3 regions correspond to vegetative cells-gate, spore-gate and microspheres-gate, respectively.

Detection limit, sensitivity and specificity of real-time PCR-based strategy to detect spores of *P. larvae* in honeys

When *P. larvae*-free honey was contaminated with *P. larvae* spores, a PCR product of expected melting temperature was observed (Fig. 3a). The assay detection limit was 2 spores g^{-1} of honey (Table 1). Below this load and also for uncontaminated honey (data not shown) no peak at 89·7°C appeared. The assay dynamic range was evidenced with honeys contaminated with tenfold serial dilutions of *P. larvae* spores (2–200 spores g^{-1} of honey). Compared to spread plate-based method, the real-time PCR-based strategy has an assay detection limit 100 times lower (200 *vs* 2 spores g^{-1} of honey, respectively) (Table 1).

Sensitivity and specificity of real-time PCR-based strategy were assessed with 14 honeys obtained from different apiaries. The expected PCR product of *P. larvae* was observed in four of them (Fig. 3b). Their spore load ranged from 63 to 135 spores g^{-1} of honey (Table 2). Only three of them were positive when tested by the spread plate method. Compared to microbiological culture, real-time PCR-based strategy has an assay sensitivity of 100% because three samples were positive for both methods, and no sample was culture positive and PCR negative. Assay specificity was 91% because 10 samples

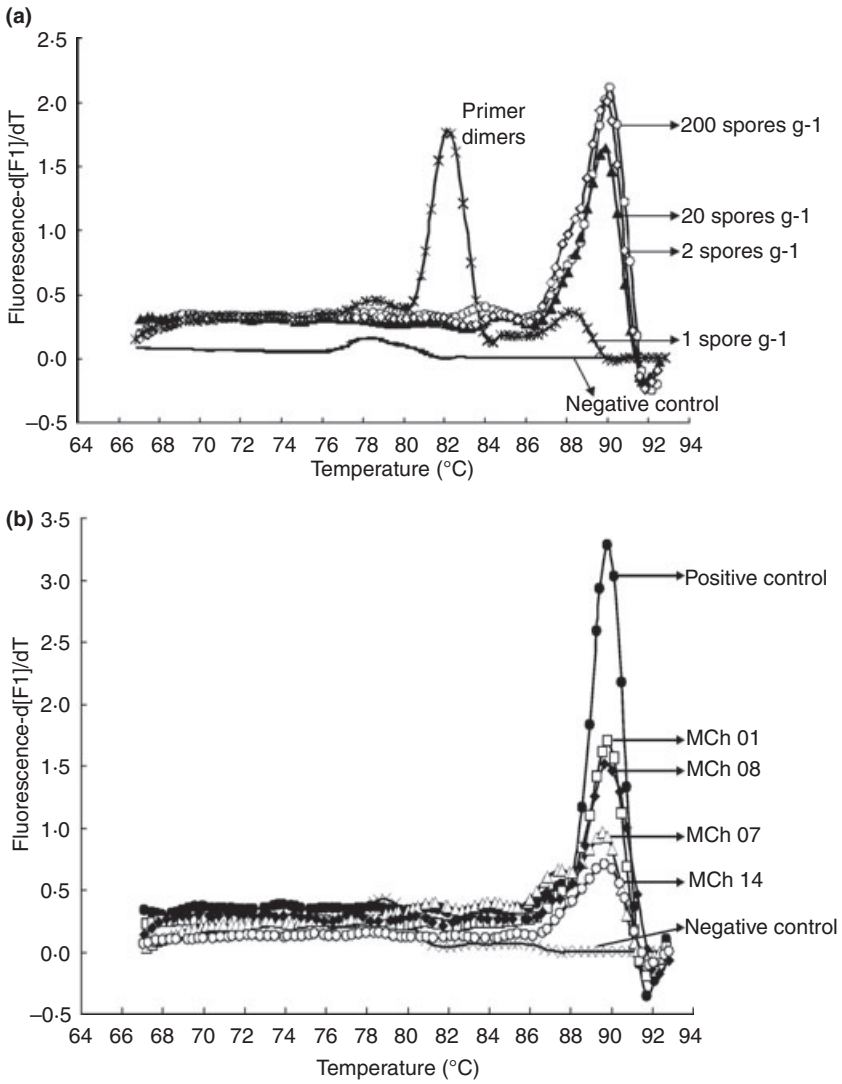

Figure 3 Detection by real-time PCR-based strategy of *Paenibacillus larvae* in experimentally and naturally contaminated honeys. Melting curves of the PCR product obtained for honeys experimentally contaminated with different amounts of *P. larvae* JM87 spores (a) or for naturally contaminated field honeys (b). Positive control corresponds to a reaction in which the template was pJMPL-2 plasmid.

were negative for both techniques, and one sample was culture negative and PCR positive. As no false-positive results were obtained, the actual assay specificity is 100%.

Discussion

The main difficulties associated with AFB control are (i) the disease vector is *P. larvae* spores, (ii) the low concentration of *P. larvae* spores in apiary supplies and products (Lindström *et al.* 2008), (iii) the low number of spores able to germinate, and (iv) the low selectivity of the *P. larvae* growth media. To circumvent these difficulties, PCR methods have been developed for *P. larvae* detection (Bakonyi *et al.* 2003; Alippi *et al.* 2004; D'Alessandro *et al.* 2007). Most of them are based on conventional PCR, which is time consuming (5–6 h) and has a relatively low technique detection limit. Furthermore, the published assay detection limits are overestimated because they have been determined against the spread plate culture method, which underestimates the number of spores. A rapid, selective, with low detection limit, sensitive and specific method to detect and quantify vegetative cells and spores of *P. larvae* is clearly required. Our real-time PCR-based strategy takes only 2 h, among other reasons because DNA extraction from spores was directly accomplished using a commercial kit. The strategy here reported clearly distinguishes between target and nontarget, closely related *Paenibacillus* and *Bacillus* species. Our technique detection limit is significantly lower than that described by Sang-Hoon *et al.* (2008) (2 *vs* 24 copies of 16S rRNA sequence per reaction, respectively). Also, our assay detection limit (0·6 spores per reaction or 2 spores g^{-1} of honey), contrasted to 20 spores per reaction (D'Alessandro *et al.* 2007), 10^5 spores g^{-1} of

Table 1 Detection of *Paenibacillus larvae* spores in experimentally contaminated honeys

Sample ID (nº of spores g^{-1} of honey)	*P. larvae* spores load	
	Spread plate (CFU g^{-1} of honey)	Real-time PCR (16S rRNA sequences)
20 000	0·20	+
2000	0·33	+
200	0·07	+
20	n.d.	+
2	n.d.	+
1	n.d.	n.d.
0·3	n.d.	n.d.
0	n.d.	n.d.

n.d., not detected.

Table 2 Detection and quantification of *Paenibacillus larvae* in field honeys

Sample ID	*P. larvae* load	
	Spread plate (CFU g^{-1} of honey)	Real-time PCR (spores g^{-1} of honey)
MCh 01	0·04	78
MCh 02	n.d.	n.d.
MCh 03	n.d.	n.d.
MCh 04	n.d.	n.d.
MCh 05	n.d.	n.d.
MCh 06	n.d.	n.d.
MCh 07	0·07	135
MCh 08	n.d.	63
MCh 09	n.d.	n.d.
MCh 10	n.d.	n.d.
MCh 11	n.d.	n.d.
MCh 12	n.d.	n.d.
MCh 13	n.d.	n.d.
MCh 14	0·07	87

n.d., not detected.

honey (Ryba *et al.* 2009) and 283 spores g^{-1} of honey (Alippi *et al.* 2004), is the lowest reported.

When compared to the spread plate method, our assay showed higher sensitivity and specificity. These differences are explained by the high detection limit of culture-based compared to PCR-based assays attributed to the fact that while PCR detect germinated and nongerminated spores, culture-based method only detect the formers (Lauro *et al.* 2003).

The key factors that explain the high performance of our strategy are the use of real-time PCR and the determination of assay detection limit with standard samples quantified by flow cytometry, which precludes the underestimation of spore loads. The capability of our strategy to detect all *P. larvae* spores is valuable because in field the proportion of germinated and nongerminated spores is unpredictable. The low detection limit of our strategy is a powerful feature as a few spores are enough for the onset of the disease in young larvae. The high selectivity and specificity of our strategy are imperative because environmental samples usually possess a high bacterial diversity. Therefore, we envisioned that our real-time PCR-based strategy to detect *P. larvae* would have significant impacts in diagnose, surveillance and prevention of AFB as it may be applied to detect *P. larvae* in adult bees, dead larvae or larval scales and also in other potential reservoirs like pollen, wax, hive walls.

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