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REPORT OF A REAL-TIME PCR ASSAY FOR PAENIBACILLUS LARVAE DNA DETECTION FROM SPORES OF SCALE SAMPLES

Reporte de un ensayo de PCR en tiempo real para la detección de ADN de Paenibacillus larvae a partir de esporas de muestras de escamas

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RESUMEN
La bacteria Gram-positiva *Paenibacillus larvae* es uno de los principales patógenos de *Apis mellifera*, causante de la enfermedad conocida como loque americana. La identificación de *P. larvae* por métodos microbiológicos consume tiempo y puede presentar dificultades. Reportamos un ensayo de PCR en tiempo real que ha sido utilizado exitosamente para la detección rápida y específica de ADN de *P. larvae* en cultivos puros y directamente desde muestras de escamas.
Palabras clave. Loque Americana, diagnóstico, *Paenibacillus larvae*, PCR en tiempo real.

SUMMARY
The Gram-positive bacterium *Paenibacillus larvae* is one of the major pathogens of *Apis mellifera*, causing the disease known as American foulbrood. Identification of *P. larvae* by microbiological methods can be difficult and time consuming. We report a real-time PCR assay that could be successfully used for rapid and specific detection of *P. larvae* DNA in bacterial pure cultures and directly from scale samples.
Key words. American foulbrood, diagnosis, *Paenibacillus larvae*, real-time PCR.

Introduction

The Gram-positive bacterium *Paenibacillus larvae* is one of the major pathogens of *Apis mellifera*, causing the disease known as American foulbrood (AFB). This condition affects the larval stage and causes, initially a ropy stage which dries down to a hard scale tightly adhering to the lower cell wall, which contains millions of bacterial spores (Genersh, 2010). Spores of the microorganism initiate the infectious stage and are the major vectors for the spread of the disease (Lindstrom et al., 2008). The delay in diagnosis contributes to colony collapse due to death of larvae, lack of progeny, and colonies death, therefore the development of a fast and reliable method of detection will be of great help to prevent the spread of the disease (Gende et al., 2011).

Diagnosis of AFB is based on identification of the pathogenic agent and the presence of clinical signs that it is usually complex and late (OIE, 2016). The confirmation of the visual AFB diagnosis requires culturing and subsequent morphological, biochemical and physiological characterization of bacterial isolates (Fernandez et al., 2010; De Graaf et al., 2013).

Molecular biology techniques play an important role in the early diagnosis of bacterial diseases, which is focused on the direct detection of target DNA sequences in samples of various matrixes (Forsgren and Laugen, 2014). DNA-based methods, such as PCR (Polymerase Chain Reaction), use specific sequences that allow the identification of bacterial species. However, conventional PCR has its limitations: the sensitivity is not high, it has poor accuracy, low resolution and it is not quantitative (Espy et al., 2006). Techniques based on conventional DNA amplification have been previously described for *P. larvae* DNA detection (Govan et al., 1999; Dobbelaere et al., 2001; Bakonyi et al., 2003; Lauro et al., 2003; Alippi et al., 2004; D'Alessandro et al., 2007; Ryba et al., 2009).

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Real-time PCR is a selective amplification of a target region within a DNA to be quantified with fluorescent markers throughout the reaction. It detects the pathogen responsible for infection with high specificity and sensitivity, rapidly (2 hours), in large scale, and allows qualitative detection and/or quantitative measurement of bacterial DNA (Burd, 2010).

Several real-time PCR assays targeting 16S rRNA for specific detection of *P. larvae* DNA have been described. Previous developed specific assays for *P. larvae* detection were applied to the evaluation of pure cultures (Han et al., 2008; Chagas et al., 2010) or to the study of infected honeybee samples (Martinez et al., 2010; Forsgren and Laugen 2014). In the case of *P. larvae* spores, their detection and quantification have been performed by the spread plate method (Leuschner et al., 2003). Although identification was made directly in putrid masses by multiplex PCR protocol (Rusenova et al., 2013), there is not background about specific *P. larvae* real-time PCR directly performed on scales samples.

The objective of this work was to develop a real-time PCR methodology with DNA extraction control for the detection of *P. larvae* DNA directly in scales samples.

Material and Methods

Pure cultures and scale samples

Collection of *P. larvae* strains and five scales samples from Research Center in Social Bees (CIAS), National University of Mar del Plata, were used. The strains had been isolated from diseased larvae of American foulbrood corresponding to apiaries from thirteen different locations in Buenos Aires province. Isolation was achieved on MYPGP agar (Dingman and Stahly, 1983), and to inhibit *Paenibacillus alvei* growth, it was supplemented with 9 µg/ml of nalidixic acid. Plates were incubated under micro aerobic conditions (5-10 % of CO_2) for 48 h at 35ºC ± 0.5ºC. Dark scale samples were taken from the remains of the diseased brood that were adhered strongly to the bottom of the comb cell. DNA was extracted from pure cultures and scales using the commercial kit AxyPrep Multisource Genomic DNA Purification (Axygen, Tewksbury MA, USA) and DNA was quantified by Quant-iT PicoGreen dsDNA Assay (Invitrogen, Carlsbad, CA, USA). All the samples analyzed showed a DNA concentration between 10 and 300 ng/µl of DNA, some were diluted for PCR amplifications at concentrations of 10-50 ng/µl, in order to avoid PCR inhibition due to excess of DNA.

DNA extraction control real-time PCR

To verify the success of DNA extraction from the scales samples and lack of inhibition in the PCR reactions DNA amplifications of *A. mellifera* beta actin gene were performed (Yang and Cox-Foster, 2005). In the case of pure cultures, as DNA extraction control, amplification with bacterial generic primers p201 (5`-GAGGAAGGIGIGGAIGACGT-3´) and p1370 (5`-AGICCCGIGAACGTATTCAC-3´) was carried out (Yang et al., 2009). The cycling program consisted of an initial denaturation of 2 minutes at 95° C, and 45 cycles of 94°C 10", 60°C 15", 72°C 15". After amplification, a melting curve analysis was performed, which resulted in single product-specific melting curve. Those scales samples with beta actin Ct (Cycle Threshold) values < 35 were considered suitable.

P. larvae real-time PCR

Specific primers to detect DNA of *P. larvae* were designed using the software Primer Premier 5 (PREMIER Biosoft International, Palo Alto, USA) based on the GenBank accession number AY030079. The designed primers (PL 167 fw 5´-CTTCGGGAGACGCCAGGTTA -3´ and PL167 rv 5´-CGCAGGCCCATCTGTAAGTG-3´) amplify a 167 bp fragment of the bacterial 16S rRNA sequence. The cycling program for detection of *P. larvae* DNA consisted of an initial denaturation of 2 minutes at 95° C, and 45 cycles of 95°C 10", 58°C 15", 72°C 15". The specific dissociation temperature of *P. larvae* PCR product was 87°C (Figure 1). During the validation process the PCR products were run on agarose gels to check the size of the amplicons. All real-time PCR reactions were carried out in a Rotor Gene thermocycler (Qiagen, Hilden, Germany) in a final volume of 20 µl using EvaGreen as intercalating fluorescent dye (KAPA FAST, Biosystems, Woburn, USA).

Assay specificity

A specificity study using as template in real-time PCR reactions DNA purified from other bacterial species: Escherichia coli (ATCC 25922), Salmonella enteriditis (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and Bacillus cereus, showed that there is no nonspecific amplification with any of the tested bacterial species.

As the genus *Paenibacillus* encompasses several species, bioinformatics tools were used to test the specifity of the designed PCR primers. We run a BLAST search (Altschul et al., 2015) and selected sequences of other different *Paenibacillus* species. A multiple sequence alignment between the different species was made, using MAFFT (Kato, 2013), corroborating the specificity of the designed primers for *P. larvae* detection (Figure 2).

Assay sensitivity

The sensitivity of the developed technique was studied with purified DNA of *P. larvae* suspensions in sterile water adjusted to 2 McFarland standard turbidity, prepared from MYPGP agar. Analyzing 1:10 serial dilutions of DNA obtained from a $2.8x10^8$ UFC/ml *P. larvae* pure culture, it was determined that the technique developed in this study shows a sensitivity of detection of 28 UFC/ml of *P. larvae* (Figure 3).

Figure 1. Melting analysis for checking the specific amplification of *P.larvae* PCR product. In the vertical axis is represented the rate of change in relative fluorescence units (RFU) with time (T) (-d (RFU) / dT) versus temperature (° C) in the horizontal axis. This rate is marked as a peak at the specific dissociation temperature of 87°C.

Figura 1. Análisis de melting para corroborar la correcta amplificación del producto de PCR de P.larvae. En el eje vertical se representa la tasa de cambio en unidades de fluorescencia relativa (RFU) con el tiempo (T) (-d (RFU) / dT) versus la temperatura en el eje horizontal. Esta tasa es marcada como un pico a la temperatura de disociación específica de 87°C.

Figure 2. Specificity analysis. Fragment of the results of a multiple sequence alignment of 16S rRNA gene sequences of different species of *Paenibacillus* (*P. larvae*, *P. koleovorans*, *P. selenitireducens*, *P. harenae*, *P. endophyticus*, *P. lupini*, *P. quercus*, *P. chibensis*, *P. relictisesami*, *P. dongdonensis*, *P. nicotianae*, *P. polymyxa*, *P. pueri*, *P. edaphicus*). The PCR primer used in this work is highlighted.

Figura 2. Análisis de especificidad. Fragmento de los resultados de un alineamiento múltiple de las secuencias del gen 16S rRNA de diferentes especies de Paenibacillus (P. larvae, P. koleovorans, P. selenitireducens, P. harenae, P. endophyticus, P. lupini, P. quercus, P. chibensis, P. relictisesami, P. dongdonensis, P. nicotianae, P. polymyxa, P. pueri, P. edaphicus). El primer de PCR utilizado en este trabajo está remarcado.

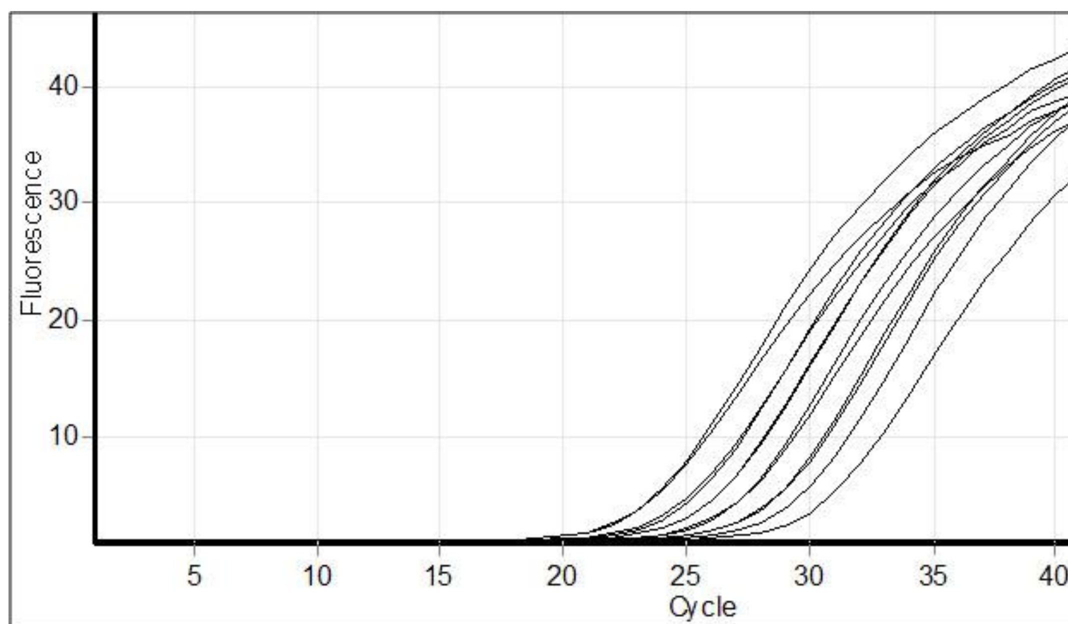

Figure 3. Protocol using as template serial dilutions of *P. larvae* purified DNA obtained from a pure culture ($2,8x10^7$, $2,8x10^6$, $2,8x10^5$, $2,8x10^4$, $2,8x10^3$, $2,8x10^2$, 28 UFC/ml). Dynamic range curve for calculating real-time PCR assay sensitivity, presented in a graphic format of increase in fluorescence (delta Rn) plotted against number of cycles.

Figure 3. *Protocolo utilizando como templado diluciones seriadas de ADN purificado de P. larvae obtenido de un cultivo puro ($2,8x10^7$, $2,8x10^6$, 2,8x105, $2,8x10^4$, $2,8x10^3$, $2,8x10^2$, 28 UFC/ml). Curva de rango dinámico para calcular la sensibilidad del ensayo de PCR, presentada como un gráfico de aumento de fluorescencia (delta Rn) en el eje vertical versus el número de ciclos en el eje horizontal.*

Results

Study of the presence of DNA from *P. larvae* in pure cultures and scales samples.

The developed technique was applied to analyze 13 *P. larvae* pure cultures and 5 scales samples, all samples were analyzed in duplicate. According to beta actin Ct values all analyzed scales samples were suitable for further study. Also, amplification with the bacterial generic primers was found in all pure cultures. *P. larvae* DNA was detected in all analyzed samples (Ct values <30). All of them, pure cultures and scales, were also analyzed by real- time PCR with the primers described by Martinez and colleagues (Martinez et al., 2010), showing all of them amplification of the specific 380 bp *P. larvae* PCR product.

Discussion

Diagnosis of AFB is based on hives visual inspection of the brood combs for clinical symptoms. The confirmation of the visual AFB diagnosis requires culturing and subsequent characterization of bacterial isolates (De Graaf et al., 2013).
We have developed a highly sensitive and specific qualitative real-time PCR assay that can be used to detect *P. larvae* DNA in spores from scales. The limit of detection of the developed technique calculated with *P. larvae* DNA dilutions was 28 UFC/ml. A specificity test was performed and showed that there is no nonspecific amplification with any of the other bacterial species tested. In addition, bioinformatic analysis was performed to corroborate no amplification of other *Paenibacillus* species.

All previously described real-time PCR techniques for *P. larvae* DNA detection lacked a DNA extraction control to detect PCR inhibition (Han et al., 2008; Chagas et al., 2010; Martinez et al., 2010; Forsgren and Laugen, 2014). In a PCR without a DNA extraction control, a negative response (no amplification) can mean that there was no target sequence present in the reaction. However, it could also mean that the reaction was inhibited due to excess of host DNA, malfunction of the thermal cycler, incorrect PCR mixture or the presence of inhibitory substances (Burd, 2010). When a PCR-based method is used in routine analysis, a DNA extraction control will indicate false-negative results (Hoofar et al., 2003). This is the first report of a *P. larvae* DNA real-time PCR detection test including a DNA extraction control. Another advantage of the developed technique is that the PCR product generated is short (167 bp), what enables the analysis of samples which DNA may be degraded, without the risk of having false negatives results.

The first step in the identification of *P. larvae* growing on solid media such us MYPGP agar, J agar, BHI agar, among

others, is the verification of its growth rate and colony morphology after, at least, two days of incubation. Later, may follow identification with other techniques (De Graaf et al., 2013).

Early detection of AFB infection is critical to avoid distribution of the infection. For this reason, rapid detection from scales without performing microbiological isolation improves the detection of AFB infections in the field.

In the present work, it was possible to develop a real-time PCR method for the detection of *P. larvae* DNA from bacterial isolates, as well as in scales samples. Thus, the real-time PCR in a few hours could determine the presence or absence of *P. larvae*. This is the first report of *P. larvae* detection by real-time PCR with DNA extraction control directly from scales.

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