Acta Scientiae Veterinariae. 38(3): 251-256, 2010.

ORIGINAL ARTICLE
Pub. 906

ISSN 1679-9216 (Online)

Detection of *Paenibacillus larvae* by Real-Time PCR

Detecção de *Paenibacillus larvae* por PCR em tempo real

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ABSTRACT

Background: *Paenibacillus larvae* is the agent of the American Foulbrood disease (AFB), which may determine the death of the hive. The detection strategy for its diagnosis is based on clinical signs of disease, isolation and identification of *P. larvae*, which usually employs microbiological and biochemical methods. Recently, molecular methods based on analysis of 16S rDNA by conventional PCR have been adopted, providing greater security and analytical speed. The rapid diagnosis is important to minimize economic losses and assess routes of spread of the pathogen. Despite the strong existing sanitary control, *P. larvae* was recently identified in the Brazilian states of Rio Grande do Sul and Paraná. After that, outbreaks have been reported in neighboring countries. This investigation was conducted to develop a protocol for detection of *P. larvae* by real-time PCR, allowing the reduction in the time of diagnosis, without loss of robustness found in the conventional PCR methods.

Materials, Methods & Results: Twenty-nine (29) *P. larvae* strains were evaluated by real-time PCR using SYBR Green. The primers *Pltr*-F/R were designed according to the sequence X60619 of 16S rDNA gene published in GenBank, to amplify a fragment of 74 base pairs. The target gene is highly conserved and specific to *P. larvae.* The amplification conditions consisted of 1 cycle of 50°C for 2 minutes and 1 cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence was monitored during the annealing at 60°C. The reactions were conducted in a 7500 Real Time PCR System equipment, using SYBRGreen PCR master mix (both Applied Biosystems), containing 2X Platinum SYBRGreen qPCR Supermix-UDG. The concentrations of primers were 1, 10 and 100 mM, and different concentrations of $MgCl_2$ (0,0mM de $MgCl_2$, 1.0mM de $MgCl_2$, 2.0mM de $MgCl_2$ and 3.0mM de $MgCl_2$) were tested, with a final volume of 50mL; 25mL and 15mL, containing a 5mL sample. The analysis of the *melting* curve was made based on a 95°C for 15 seconds and 60°C for 20 seconds and one cycle with temperature ranging between 60°C and 95°C for 20 minutes. The best results of sensitivity and specificity in the reaction with SYBR Green were obtained with primer concentration set as 100 mM. The different concentrations of $MgCl_2$ tested did not affect the performance of the reaction. No amplification was observed with DNA obtained from *Paenibacillus alvei* or *Bacillus* species. The limit of detection was set as 6 pg of DNA template. The regression analysis of the C_T values of the PCR products showed a linear relationship between the initial amounts of DNA template and the values of C_T ($R^2 = 0.9982$), indicating that the test is highly precise.

Discussion: The protocol developed allowed the unequivocal identification of *P. larvae,* as all strains were detected by this approach. The amplification of the expected 16S rDNA gene fragment was verified by amplification with the primers *Pltr* F/R only for chromosomal DNA of *P. larvae.* In addition, the amplicon specificity was verified by sequencing and no amplification was observed when the primers were tested with DNA from other bacterial species. The protocol developed in this study proved to be sensitive and specific, providing a rapid and accurate diagnostic tool. The results showed that the analysis by real-time PCR of partial 16S rDNA gene of *P. larvae* represents an important alternative for rapid diagnosis of AFB disease. The use of this methodology may represent an advance for rapid confirmation of the presence of this bacterium, what will allow the adoption of control measures against AFB, which can avoid its spreading in Brazilian territory.

Descritores: *Paenibacillus larvae,* diagnóstico, 16S rDNA, PCR em tempo real.

Keywords: *Paenibacillus larvae,* diagnosis, 16S rDNA, real-time PCR.

Received: November 2009 www.ufrgs.br/actavet Accepted: January 2010

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INTRODUCTION

The *American Foulbrood* (AFB) is a notifiable disease of high economic importance. His causative agent called *Paenibacillus larvae* only attacks the larval stage of the bee *Apis mellifera* and other *Apis* spp [6,21]. Infection occurs by ingestion of honey contaminated with spores [2,14], that after 24 hours germinate in the intestine and spread, producing septicemia and death [6]. The clinical symptoms of the disease are typical, and the affected larvae have a dark and viscous aspect [6,20]. Bees from infected hives and even beekeepers can contribute to the spread of the disease. The detection of *P. larvae* is performed using microbiological culture [18], phenotypical characteristics [9,13], or molecular diagnostic methods based on comparative analysis of sequences of the 16S rDNA gene [5,7,10].

In South America, the first isolation of *P. larvae* occurred in 1989 in Argentina [1], and afterwards in Uruguay [4]. In Brazil, despite efforts to prevent its introduction, *P. larvae* was detected in 2002 in the State of Rio Grande do Sul, in beehives without clinical signs of disease [19]. Recently it was reported an outbreak in hives in the State of Paraná [12], motivating the development of alternative detection methods. Molecular Methods based on the detection of *P. larvae* by PCR have been described [7,10], including a real-time PCR procedure [8]. Real-time PCR is characterized by its high accuracy and reproducibility, and precision in data collection depends on factors such as the PCR conditions, sample preparation and quality of DNA [22]. In contrast to conventional PCR, which is based on amplification of DNA with subsequent electrophoresis and visualization, real-time PCR is automated, highly sensitive, robust and does not require procedures for post-PCR identification [11].

This study aimed to develop a protocol for detection of *P. larvae* by real-time PCR to allow the reduction in the time of diagnosis, without loss of robustness found in the reactions by conventional PCR.

MATERIALS AND METHODS

Microorganisms

In this study, 28 isolates of *Paenibacillus larvae* from the collection of National Agricultural Laboratory (LANAGRO/RS) were used. *P. larvae* ATCC 9545 was used as positive control, and *Paenibacillus alvei*, *Bacillus cereus* var. *mycoides* ATCC 11778, and *Bacillus anthracis* Sterne were used as negative controls. Samples of *P. larvae* and *P. alvei* were grown on *P. larvae* agar plates [18], incubated at 35°C and monitored for 5 days. *B. cereus* var. *mycoides* ATCC 11778 was cultivated in BHI, incubated at 37°C and monitored for 5 days.

Primers

The forward primer (*Pltr*-F 5'GGAGTGACG GTACTTGAGAAGAAAG3') and reverse (*Pltr*-R 5'CGCTTGCCCCCTACGTATTA3') were selected according to the sequence X60619 of 16S rDNA gene published in GenBank, with the help of the software Primer Express[1]. The target gene is highly conserved and specific to *P. larvae*. The primers amplified a fragment of 74 base pairs.

Preparation of DNA

A colony was isolated from each sample was suspended in 100 µL of distilled water and subjected to the DNA extraction protocol for bacteria by using phenol-chloroform [17]. The DNA was resuspended in 50 µL of buffer consisting of 10 mM Tris/HCl pH 8.0, containing 1 mM EDTA, and quantified using a UV spectrophotometer[2] at wavelengths of 260 and 280 nm.

Real-time PCR

Twenty-nine (29) *P. larvae* strains were used to evaluate the performance of real-time PCR using SYBR Green[1]. The amplification conditions consisted of 1 cycle of 50°C for 2 minutes and 1 cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence was monitored during the annealing at 60°C. The reactions were conducted in an equipment 7500 Real Time PCR System using SYBR Green PCR master mix[1] containing 2X Platinum SYBR Green qPCR Supermix-UDG. The concentrations of primers tested were 1, 10 and 100 µM, and different concentrations of $MgCl_2$ (0, 1.0, 2.0 and 3.0 mM) in a final volume of 50 µL, 25 µL and 15 µL, containing 5 µL of sample, were tested.

The specificity of the primers was verified by comparing the partial sequence of the 16S rDNA of *P. larvae* with the sequences of other organisms listed in Table 1 using the BLAST algorithm [3], and by reaction with DNA extracted from the other bacterial species used as controls.

The analysis of the *melting* curve (T_M) was made based on a 95°C for 15 seconds and 60°C for

Table 1. Sequences of 16S rDNA used to confirm the specificity of primers *Pltr* F/R by means of BLAST algorithm.

Organism	GenBank accession number	16S rDNA lenght (bp)
Paenibacillus larvae	AY030079	1389
Paenibacillus peoriae	D78476	1437
Paenibacillus glucanolyticus	D88514	1414
Paenibacillus alvei	X60604	1434
Paenibacillus koreensis	AF130254	1536
Paenibacillus aliginolyticus	D78465	1424
Paenibacillus azotofixans	AJ251192	1484
Paenibacillus polymyxa	AY359637	1440

20 seconds and one cycle with temperature ranging between 60°C and 95°C for 20 minutes.

To determine the sensitivity of the reaction, *P. larvae,* in the concentration of 10^5 CFU/mL, had their DNA extracted, sequentially diluted to the quantity of 0.6 pg and subjected to analysis by real-time PCR in the same conditions of the other samples.

RESULTS

In the evaluation of different amounts of primers *Pltr* F/R, the best results of sensitivity and specificity in the reaction with SYBR Green were obtained with 100 µM. The reaction was optimized and the reaction mix was composed of 1.5 µL of DNA, 7.5 µL of SYBR Green Platinum qPCR Supermix-UDG (2X), 0.03 µL of Rox, 100 µM of each forward and reverse primer, a final volume of 15 µL. The different concentrations of $MgCl_2$ tested did not affect the performance of the reaction. The detection of 16S rDNA gene was proven by ampli-fication of fragments of expected sizes when the primers *Pltr* F/R were tested with samples of *P. larvae*. All 29 samples of *P. larvae* (1 control strain and 28 test strains) responded to this approach. The primers *Pltr* F/R were highly specific for *P. larvae*, as observed by the absence of cross-reactivity with DNA samples from other bacterial species. In the other species tested, no amplification was observed. The specificity was also verified by sequencing of the PCR products, which shared 100% identity with the corresponding sequence of 16S rDNA of *P. larvae*.

The reaction was optimized using 60 ng of purified DNA per reaction. In the 29 samples that were amplified by this approach, detection of the product resulting from amplification of partial 16S rDNA gene occurred in the C_T 20 (mean 19.5 ± 0.4). In establishing the limit of detection (LOD), initial quantities of *P. larvae* chromosomal DNA template were serially diluted 10-fold from 60 ng to 0.6 pg. DNA at a concentration of 60 ng was detected in 18 C_T. Consecutive dilutions resulted readings of fluo-rescence in C_T 21, 24, 27 and 30, showing a linear relationship with the DNA amount in the range of 60 ng to 6 pg (Figure 1). LOD was established as 6 pg.

After amplification, the analysis of disso-ciation products PCR was performed. The specificity of the primers and the absence of nonspecific products can be observed in the analysis of the melting curve. The values of the melting temperature were between 83.2°C and 83.9°C. No amplification (C_T e ≥ 40) was observed with DNA obtained from *Paenibacillus alvei* or from other *Bacillus* species. It was found that the melting temperature was 83.3°C ± 0.5 for the gene fragment 16S rDNA, confirming the specificity of the reaction (Table 2). The results confirm that the test developed with SYBR Green is 100% specific, detecting only strains of *P. larvae*.

DISCUSSION

In the present study, a real-time PCR protocol was developed, allowing the unequivocal identi-

fication of *P. larvae*. The results of PCR reactions also demonstrated the high sensitivity of the protocol developed for detection of *P. larvae*, since amplification of 6 pg of chromosomal DNA was observed.

The results agreed with those observed by conventional diagnosis, confirming the expected observations for *P. larvae,* [19]. The real-time PCR protocol developed for the detection of *P. larvae*, adjusted to allow its maximum performance, indicates that the time for diagnosis of AFB could be reduced as compared with conventional PCR methodologies (Table 3). In addition, real-time PCR is about 100 times more sensitive than conventional PCR methods [16,22], which need post-PCR steps for product evaluation. Some conventional PCR protocols for identification of *P. larvae* have been described in the past decade. Those methods are often based on detection of 16S rDNA sequences, using *P. larvae* from pure cultures, larvae, adult bees and inoculated honey [2,5]. Some protocols shown elevated specificity and allow detection of *P. larvae* below pathogenic levels [10]. The PCR protocol using the primers *Pl4* and *Pl5*, which amplify a 700 bp sequence of 16S rDNA, allows the detection of the PCR product at a 10^{-2} dilution of template DNA obtained from 32 spores [15]. However, no similar study to detect *P. larvae* by real-time PCR was conducted to date. Recently, a microchip-based real-time PCR technique, using a thermal cycler specially designed to withstand microvolume reaction and conducted in glass and silicon microchips, was effec-tively used to detect *P. larvae* [8] in 8 minutes in a volume of 6.0 µL. These are some fundamental differences in comparison with the protocol presented in this work, which used standard real-time PCR equipment.

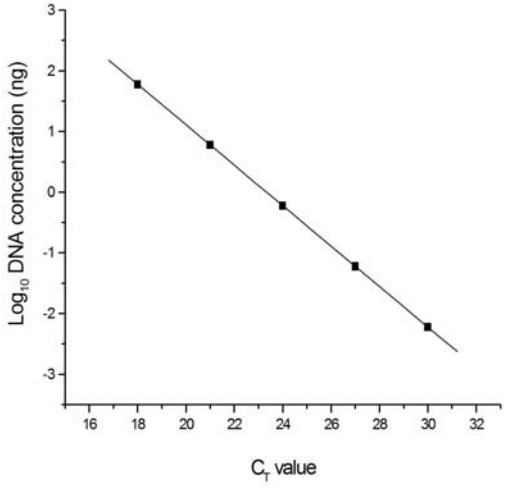

Figure 1. Relationship between serial dilutions of chro-mosomal DNA from *Paenibacillus larvae* ATCC 9545 and the threshold cycle. The C_T values increased in proportion to initial DNA template quantities in the range of 60 ng - 6 pg. Data were subjected to linear regression analysis. The correlation coefficient (R^2) was 0.9982.

The ability to test real-time PCR to determine the precise number of copies of DNA present in the sample depends on the linearity and efficiency of the test. The values of $R^2 = 0.9982$ obtained with SYBR Green indicates that the test is highly linear. In addition, the estimated value of the slope of the linear regression (slope -3.2509) is similar to the theoretical optimum of -3.32 using purified DNA and calibrated cell suspensions [11].

The regression analysis of the C_T values from the products of real-time PCR shows a linear relationship between the initial amounts of template and the values of C_T (R^2 = 0.9982), which increased as the DNA concentration decreased. All products are considered identical, as can be evidenced by analysis of melting temperature, because the temperature variation between them is small and there are no particular products [22]. The observed lowest amount of analyte that can be detected (LOD), but not necessarily quantified, was set as 6 pg of DNA template. Since the infected larvae usually die from the AFB disease with about 10^9 bacterial spores/ml [6], this LOD value might be acceptable for the application on environmental honey bee larva samples and the methodology might be capable to detect the pathogen in the early stage of the infection.

CONCLUSIONS

The real-time PCR protocol developed in this study proved to be sensitive and specific, providing

Table 2. Sensitivity of the primers *Pltr* F/R for detection of *Paenibacillus larvae* ATCC 9545 by real-time PCR.

DNA amount (ng)	C_T (cycle)[a]	Tm (ºC)[b]
60	18	83.3
6	21	83.6
0.6	24	83.6
0.06	27	83.2
0.006	30	83.9

[a]The threshold cycle.
[b]The melting temperature of amplicon.

a rapid and accurate diagnostic tool. The primers *Pltr* F/R enable the specific amplification of a 16S rDNA sequence of *P. larvae* in 54 minutes, with a detection limit of 6 pg of chromosomal DNA per reaction. This methodology may be useful to a rapid confirmation of the presence of *P. larvae*, allowing the adoption of prophylactic control measures against AFB that can avoid its establishment in Brazilian territory.

SOURCES AND MANUFACTURERS

[1]Applied Biosystems, Foster City, CA, USA.
[2]Specord 40, Analytik Jena, Jena, Germany.

Table 3. Comparison of detection of *Paenibacillus larvae* by conventional PCR and real-time PCR based on amplification of 16S rDNA sequences.

Primer[a]	Sequence 5' - 3'	Amplicon	PCR type	Running time (min)	Reference
Prim1, 2F	AAGTCGAGCGGACCTTGTGTTTC	973	Conventional[b]	86	[7]
Prim1, 2R	GGAGACTGGCCAAAACTCTATCT				
*Ple*F	TCGAGCGGACCTTGTGT	969	Nested-PCR[c]	160	[10]
*Ple*R	CTATCTCAAAACCGCTCAGAG				
*Pli*F	CTTCGCATGAAGAAGTCATC	572			
*Pli*R	TCAGTTATAGGCCAGAAAGC				
*Pltr*F	GGAGTGACGGTACTTGAGAAGAAAG	74	Real-time[d]	54	Present
*Pltr*R	CGCTTGCCCCCTACGTATTA				article

[a] F and R correspond to the forward and reverse primers, respectively.
[b] The PCR conditions were 95ºC (1 min), 30 cycles 93ºC (1 min), 55ºC (30 s), 72ºC (1 min), and 72ºC (5 min).
[c] For the amplification of external primers *Ple* the annealing temperature was lowered 0.5ºC/cycle, from 69 to 59ºC, with each annealing step lasting 30 s. Denaturation and extension steps were all executed at 94ºC (30 s) and 72ºC (45 s), respectively. Cycling conditions for internal primers *Pli* were 94ºC (30 s), 59ºC (30 s), 72ºC (45 s) for 30 cycles followed by 5 min at 72ºC.
[d] The PCR conditions were 50ºC (2 min), 95ºC (2 min), 40 cycles 95ºC (15 s), 60ºC (1 min).

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