

## Development and Evaluation of PCR Assays for the Detection of *Paenibacillus larvae* in Honey Samples: Comparison with Isolation and Biochemical Characterization

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PCR assays were developed for the direct detection of *Paenibacillus larvae* in honey samples and compared with isolation and biochemical characterization procedures. Different primer pairs, designed from the 16S rRNA and the metalloproteinase precursor gene regions, and different DNA extraction methods were tested and compared. The sensitivity of the reactions was evaluated by serial dilutions of DNA extracts obtained from *P. larvae* cultures. The specificity of the primers was assessed by analyzing related *Paenibacillus* and *Bacillus* strains isolated from honey. The PCR assays also amplified these related bacteria, but at lower sensitivity. In the next step, the PCR assays were applied to contaminated honey and other bee products originating from 15 countries. Lysozyme treatment followed by proteinase K digestion was determined to be the best DNA extraction method for *P. larvae* spores. The most sensitive primer pair detected *P. larvae* in 18 of 23 contaminated honey samples, as well as in pollen, wax, and brood. Honey specimens containing saprophyte bacilli and paenibacilli, but not *P. larvae*, were PCR negative. Although the isolation and biochemical identification method (BioLog) showed higher sensitivity and specificity, PCR proved to be a valuable technique for large-scale screening of honey samples for American foulbrood, especially considering its rapidity and moderate costs.

American foulbrood is a disease affecting the larval stage of the honeybee (*Apis mellifera* L.). The causative agent is *Paenibacillus larvae* subspecies *larvae*, a gram-positive, spore-forming bacterium which infects only bees. In consequence of its destructive effects, extreme resistance, and worldwide distribution, *P. larvae* is considered to be one of the most important infectious agents of the honeybee. Thus, American foulbrood is a notifiable disease of high socioeconomic importance and significance in international trade (a list B disease according to the classification of the Office International des Epizooties).

*P. larvae* (White) subsp. *larvae* is a slender rod about 2.5 to 5 µm long and about 0.5 µm wide. The bacterium forms large numbers of oval spores in infected larvae within a few days. The spores are highly resistant to heat and chemical agents and can survive in the environment for several years (12). Only the spores are capable of inducing disease.

This bacterium, formerly named *Bacillus larvae*, was reclassified into the new genus *Paenibacillus* (5) on the basis of the 16S rRNA sequence diversity. The *P. larvae* strains are divided into two distinct subspecies, *P. larvae* subsp. *larvae* and *P. larvae*

subsp. *pulvificiens* (14). Although *P. larvae* subsp. *larvae* is considered to be the cause of American foulbrood, *P. larvae* subsp. *pulvificiens* is capable of causing similar but usually milder clinical symptoms.

The spread of the agent between apiaries or countries is often associated with the import of honeybee colonies or the use of contaminated honey (6, 11, 17, 26) for feeding purposes. In most cases, American foulbrood can be diagnosed in the field by visual inspection; however, the diagnosis must be confirmed by laboratory tests. Microscopic identification of stained bacteria can be complemented by the modified hanging-drop technique (19), the Holst milk test (15), or fluorescent-antibody techniques developed for the detection of *P. larvae* antigens (20, 25, 27). Besides microscopy, the most frequently applied identification method is the isolation of *P. larvae* using culture media followed by characterization by biochemical tests. The bacterium requires complex media for growth (24). For biochemical diagnosis of suspected *P. larvae* colonies, the catalase (13) and nitrate reduction (18) tests are widely used.

Detection of *P. larvae* in honey samples is a valuable method of control of the spread of the disease within and between countries (17, 26). The major problems associated with the diagnosis of *P. larvae* in honey specimens are the relatively low concentration of *P. larvae* spores in the honey and the presence of related *Paenibacillus* or *Bacillus* species, which often over-

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grow the *P. larvae* colonies. Spores can be purified and concentrated by dialysis (22) and/or centrifugation (16) of the diluted honey samples. Heat treatment is used for the inactivation of non-spore-forming bacteria. Several selective media have been developed for the cultivation of *P. larvae*, such as sheep blood agar containing nalidixic acid (16) or J agar (10) with nalidixic acid and piperimidic acid (1). A virulent mutant bacteriophage from *P. larvae* has also been used for the identification of *P. larvae* in honey samples (23). Biochemical identification and characterization systems (e.g., BioLog) are appropriate methods for the recognition of *P. larvae* as well.

Molecular techniques have also been developed for the identification of *P. larvae* (2, 3). Govan et al. (8) have described a PCR assay for the detection of *P. larvae*-specific DNA in bacterial colonies grown on semiselective medium. This technique was suggested for use in the rapid confirmation of the presence of *P. larvae* strains isolated from honey samples. Dobelaere et al. (7) also described a PCR assay for the identification of cultivated bacteria and for the detection of *P. larvae* in DNA extracts of the remains of American foulbrood-diseased larvae. Very recently, Alippi et al. (4) described a method, based on PCR and restriction fragment analysis, which allows the differentiation of *P. larvae* subsp. *larvae* strains from all other bacterial species.

The aim of this study was to develop a sensitive and specific PCR assay for the direct detection of *P. larvae* in contaminated honey samples. Different techniques for the isolation of DNA from bacterial spores were compared, and different sets of oligonucleotide primers were evaluated for their sensitivity and specificity. The PCR assays were compared to the "gold standard" of *P. larvae* cultivation and biochemical identification methods (BioLog).

#### MATERIALS AND METHODS

**Samples.** Cultures of *P. larvae* subsp. *larvae*, *P. larvae* subsp. *pulvificiens*, *P. alvei*, *Bacillus cereus*, *B. megaterium*, and *B. subtilis* on Columbia blood agar, contaminated honey, pollen, wax samples, and infected brood were investigated. The *Paenibacillus* strains were isolated from honey samples collected in Australia (1 sample), Austria (10 samples), China (1 sample), France (2 samples), Greece (3 samples), Hungary (1 sample), Iran (1 sample), and Romania (1 sample) within a 6-year period. The honey, pollen, and wax samples were tested for the presence of *P. larvae* by cultivation. For the origin of the honey samples and other bee products, see Table 4.

**Sample processing.** Bacterial colonies were collected with a sterile inoculation loop and were dispersed in 500  $\mu$ l of phosphate-buffered saline. A 100- $\mu$ l volume of the bacterial suspension was centrifuged at 20,000  $\times g$  for 10 min, and the pellet was used for DNA isolation. For honey samples, 10 g of honey was diluted in 10 ml of sterile distilled water and the solutions were incubated at 95°C for 6 min. Thereafter, 10 ml of each solution was centrifuged at 4,000  $\times g$  for 30 min and the pellet was used for DNA extraction. The pollen and wax samples were processed similarly, but the initial amount of sample was reduced to 1 g. For testing infected brood, larvae showing clinical signs were homogenized in 500  $\mu$ l of phosphate-buffered saline; 100  $\mu$ l of homogenate was centrifuged (14,000  $\times g$  for 10 min), and the pellet was used for further analysis.

**DNA extractions.** Three different nucleic acid isolation methods were tested comparatively: guanidium thiocyanate treatment (method A), cetyltrimethylammonium bromide treatment (method B), and lysozyme treatment (method C).

(i) **DNA extraction method A.** The bacterial pellets were resuspended in denaturing buffer containing guanidinium thiocyanate, *N*-lauroylsarcosine, sodium citrate, and 2-mercaptoethanol. After mixing, sodium acetate was added, and then nucleic acid was extracted with phenol-chloroform-isoamyl alcohol. The nucleic acids were precipitated with isopropanol, and the precipitates were resolved in elution buffer (Qiagen).

(ii) **DNA extraction method B.** The bacterial pellets were resuspended in lysis buffer containing cetyltrimethylammonium bromide, Tris-HCl, NaCl, EDTA,

and 2-mercaptoethanol. After vortexing, the mixtures were frozen in liquid nitrogen and thawed by shaking at room temperature; this procedure was repeated twice. Thereafter, nucleic acid was extracted with phenol-chloroform-isoamyl alcohol and precipitated with isopropanol. The precipitates were resolved in elution buffer (Qiagen).

(iii) **DNA extraction method C.** The bacterial pellets were resuspended in 200  $\mu$ l of enzyme solution containing 20 mg of lysozyme per ml, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1.2% Triton and incubated for 1 h at 37°C. Then 25  $\mu$ l of proteinase K and 200  $\mu$ l of buffer AL (Qiagen) were added, and the lysates were incubated at 56°C for 30 min and at 96°C for 5 min. Bacterial DNA was isolated using the QIAamp DNA minikit (Qiagen) as specified by the manufacturer. DNA was eluted with 200  $\mu$ l of elution buffer and stored at -20°C.

**PCR.** Specified DNA fragments of *P. larvae* and other *Paenibacillus* and *Bacillus* species were amplified using PCR.

(i) **Primer design.** Oligonucleotide primers were designed on the basis of the published nucleotide sequences of *P. larvae*. The primers were constructed with the help of the Primer Designer program (Scientific and Educational Software, version 3.0). A previously described *P. larvae*-specific primer pair (PL 1-PL 2) (8) was also included in our investigations. The primer sequences, orientations, origins, locations, and resulting product sizes are shown in Table 1. Nucleotide positions refer to the *P. larvae* sequences with accession no. X60619 and AF111421. The primers were synthesized by GibcoBRL Life Technologies, Ltd. (Paisley, Scotland).

(ii) **Amplification.** The 25- $\mu$ l reaction mixtures contained 2.5  $\mu$ l of 10 $\times$  reaction buffer (Perkin-Elmer) which included MgCl<sub>2</sub> (final concentration, 1.5 mM), 2 mM deoxynucleoside triphosphate mix, 0.8  $\mu$ M (each) appropriate forward and reverse primers, 0.625 U of *Taq* DNA polymerase (Promega), and 5  $\mu$ l of template DNA. Following an initial denaturation step at 95°C for 5 min, the reaction mixtures were subjected to 40 cycles of heat denaturation at 95°C for 20 s, primer annealing at 50°C for 20 s, and DNA extension at 72°C for 1 min. The reactions were completed by a final extension of 7 min at 72°C. The amplifications were performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The amplicons were stored at 4°C until electrophoresis was carried out.

**Gel electrophoresis.** Samples (5  $\mu$ l) of the amplicons were electrophoresed in a 1.2% Tris acetate-EDTA-agarose gel at 6 V/cm for 80 min. The gel was stained with ethidium bromide, and the bands were visualized under UV light at 312 nm using a TFX 35M UV transilluminator (Life Technologies) and photographed with a Kodak DS electrophoresis documentation and analysis system using the Kodak Digital Science 1D software program. Product sizes were determined by reference to a 100-bp molecular size ladder (Amersham Pharmacia Biotech).

**Nucleotide sequencing and computer analysis.** To confirm the specificity of the PCR assays, PCR amplification products of five selected honey samples positive for *P. larvae* subsp. *larvae* and one sample of cultivated *P. alvei* were sequenced, and the sequences were aligned with the published sequences available in the GenBank database. The method of fluorescence-based sequencing has been described previously by our group (9).

**Culture of bacteria.** The bacteria from the aforementioned samples were isolated, identified, and characterized on the basis of their biochemical properties. For the honey samples, 5 g of honey was diluted in 5 ml of sterile distilled water and the solutions were incubated at 90°C for 6 min to inactivate non-spore-forming bacteria. From each sample, 400  $\mu$ l of diluted honey was inoculated onto each of six Columbia sheep blood agar plates. The plates were incubated at 37°C and were checked for *P. larvae* growth on days 3 and 6. The bacterial colonies were isolated, and the bacteria were characterized by light microscopy following Gram stain, catalase test (13) (*P. larvae* is catalase negative), and formation of great whips in the fluid part of Columbia blood slant agar (21).

**Metabolic fingerprint technique.** Further identification and characterization of the bacterial isolates were performed using the BioLog GP MicroPlate test panel. The tests were carried out as specified by the manufacturer. The plates were read visually after 4 and 24 h. The results were evaluated using the MicroLog2 system (Biolog, Inc.).

#### RESULTS

**PCR assays applied to cultured bacteria.** Specific DNA fragments of *P. larvae* subsp. *larvae* were amplified by PCR assays with nine different primer pairs (Table 1). The primers were first tested on a *P. larvae* subsp. *larvae* strain isolated in Austria (DSMZ 7030). Amplifications with the selected primer pairs

TABLE 1. Oligonucleotide primers selected for *P. larvae* PCR

Primer <sup>a</sup>	Sequence (5' to 3')	Origin	Nucleotide positions	Product length (bp)
<b>AF 1f</b>	<b>GCT CTG TTG CCA AGG AAG AA</b>	16S rRNA	436–455	451
<b>AF 2r</b>	<b>AGG CCG AAT GCT TAC TGT GT</b>		868–887	
AF 3r	TGT CAC CGG CAG TCA TCT TA	16S rRNA	1156–1175	739 <sup>b</sup>
AF 4f	CTG GCG GCG TGC CTA ATA CA	16S rRNA	30–49	374
AF 5r	GGC GTT GCT CCG TCA GAC TT		385–404	
<b>AF 6f</b>	<b>GCA AGT CGA GCG GAC CTT GT</b>	16S rRNA	51–70	237
<b>AF 7r</b>	<b>GCA TCG TCG CCT TGG TAA GC</b>		269–289	
AF 8f	GGA GTA ACT GCC CCT GGA GT	16S rRNA	464–483	695
AF 9r	CTT AGA GTG CCC ACC TCT GC		1140–1159	
AF 10f	TAG GCC ATG AAT TGA CTC AC	Metalloproteinase	89–108	155
AF 11r	TAT TCG GCG TAT ACA CGT CT		225–244	
Paeni 30f	CGT ATT CAG AGA CGG TGA TG	Metalloproteinase	30–49	242
Paeni 253r	TCT AAG GAA CGG AGA GCA TC		253–272	
PBL 8f	CTT TCT GGA ACG GGC AGC AA	Metalloproteinase	8–27	342
PBL 350r	GTA TGA ACG CCG CCG TTA TC		331–350	
PL 1f <sup>c</sup>	AAG TCG AGC GGA CCT TGT GTT TC	16S rRNA	53–75	971
PL 2r <sup>c</sup>	TCT ATC TCA AAA CCG GTC AGA GG		1002–1024	

<sup>a</sup> f, forward primer; r, reverse primer. The primer pairs which gave the best results are highlighted in bold.

<sup>b</sup> With the AF 1f primer.

<sup>c</sup> Primers published by Govan et al. (8).

resulted in PCR products with the expected molecular weights. The electrophoresis pattern of the amplicons is presented in Fig. 1. Another 19 *P. larvae* subsp. *larvae* strains isolated from bee products originating from eight countries were tested by PCR. All strains proved to be clearly positive in the assays (data not shown).

To reveal the specificity of the reactions, *P. larvae* subsp. *pulvifaciens*, *P. alvei*, *B. cereus*, *B. megaterium*, and *B. subtilis* strains were also tested with the selected primer pairs. PCRs applied to undiluted bacterial DNA extracts resulted in amplification products in all involved *Paenibacillus* and *Bacillus* species with all primer pairs. The extent of cross-reactivity of the PCR assays with the different *Paenibacillus* and *Bacillus* species was determined by titration of the DNA extracts. Tenfold serial dilutions were prepared, and PCRs were performed on these diluted bacterial strains. For this experiment, the primer pairs AF 1 plus AF 2, AF 6 plus AF 7, PBL 8 plus PBL 350, and

PL 1 plus PL 2 were selected based on preliminary evaluations (data not shown).

The AF 1 plus AF 2 primers detected *P. larvae* (subsp. *larvae* and *pulvifaciens*) DNA up to a dilution of  $10^{-8}$ , which contained approximately 0.9 to 1.1 CFU of bacteria. The other bacterial species were positive only up to dilutions containing  $8 \times 10^6$  CFU (*B. subtilis*),  $7 \times 10^5$  CFU (*B. cereus* and *B. megaterium*), and  $9 \times 10^4$  CFU (*P. alvei*), respectively (Fig. 2). The AF 6 plus AF 7 primers detected *P. larvae* DNA even at a sensitivity level of 0.05 CFU (*P. larvae* subsp. *larvae*) and 0.1 CFU (*P. larvae* subsp. *pulvifaciens*), respectively; the other tested bacterial species, however, also gave a positive reaction with the AF 6 plus AF 7 primer pair, up to a sensitivity of 2.6 to 7 CFU of bacteria in one case (*P. alvei*). The other primers detected *P. larvae* only when large amounts ( $10^5$  to  $10^7$  CFU) were present, and in some cases nonspecific bands of different sizes were produced when other bacterial species were tested (data not shown).

**PCR assays applied to honey and other bee products.** PCR assays were performed on 26 honey samples originating from 13 different countries (on four continents) to detect *P. larvae*-specific DNA (see Table 4). Two pollen samples, one wax sample, and one brood sample were also tested.

Because of the resistance of *Paenibacillus* spores to proteolytic enzymes, three different DNA isolation methods were tested in parallel on seven randomly selected honey samples and on one pollen specimen. The results of the comparison of the DNA isolation techniques are presented in Table 2. All three methods were effective in seven of eight samples; however, following PCR, the sharpness of the bands and the amount of amplified PCR products differed significantly: the largest amount of amplified DNA was obtained when method C was used for DNA extraction. Consequently, extraction method C was applied to all other samples.

Based on the results of the aforementioned sensitivity tests of the different primers, four primer pairs were compared using DNA extracts of 14 randomly selected honey samples.

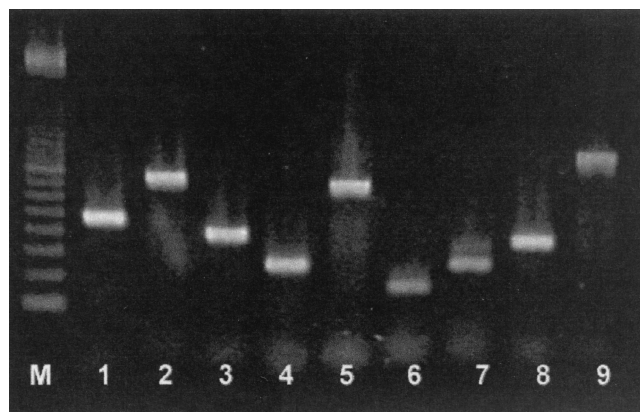


FIG. 1. Gel electrophoresis of PCR products of *P. larvae* subsp. *larvae* amplified with different primer pairs. Lanes: 1, AF 1 plus AF 2; 2, AF 1 plus AF 3; 3, AF 4 plus AF 5; 4, AF 6 plus AF 7; 5, AF 8 plus AF 9; 6, AF 10 plus AF 11; 7, Paeni 30 plus Paeni 253; 8, PBL 8 plus PBL 350; 9, PL 1 plus PL 2; M, molecular size marker (100-bp ladder).

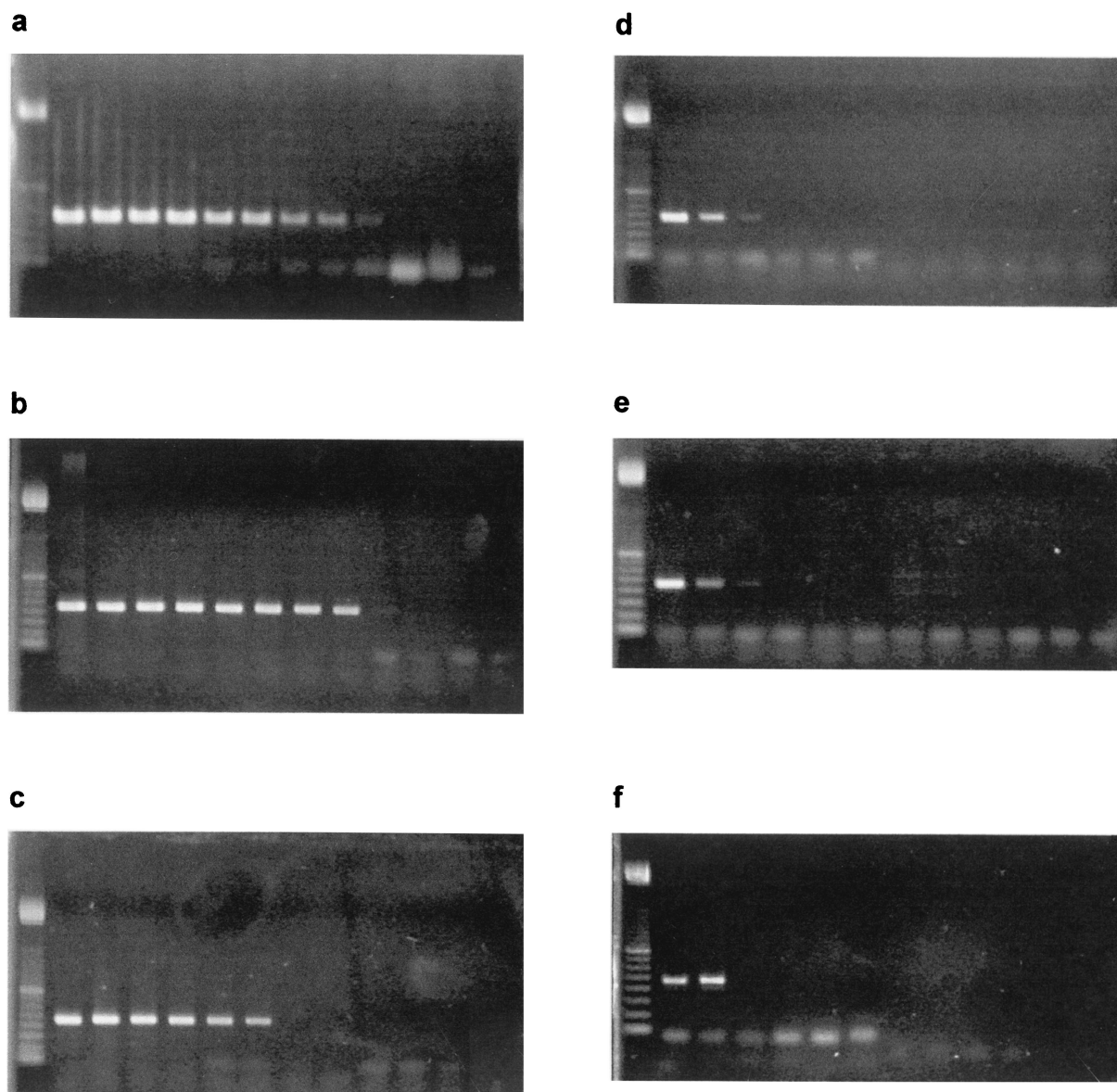


FIG. 2. Gel electrophoresis of PCR products from serial dilutions of DNA extracts from different bacteria using the AF 1 plus AF 2 primer pair. (a) *P. larvae* subsp. *larvae*; (b) *P. larvae* subsp. *pulvifaciens*; (c) *P. alvei*; (d) *B. cereus*; (e) *B. megaterium*; (f) *B. subtilis*. Lanes (from left to right): molecular size marker (100-bp ladder), undiluted DNA, 10-fold dilution steps  $10^{-1}$  to  $10^{-10}$ , negative control.

TABLE 2. Comparison of different DNA extraction methods prior to PCR

Sample	Code	Type of sample	Extraction efficiency of:		
			Method A	Method B	Method C
1	2001/36	Honey	-	+	+
3	2001/89	Honey	+	-	+
9	AZ2000/696	Honey	+	+	+
12	AZ2000/691	Honey	+	+	-
14	AZ2000/1112	Honey	+	+	+
17	AZ95/22	Honey	+	+	+
22	BP31/2001	Honey	+	+	+
28	BP79/2000	Pollen	+	+	+

The results are presented in Table 3. Amplifications with the AF 1 plus AF 2 primers were positive in 8 of 14 samples, while the AF 6 plus AF 7 primers detected *P. larvae* DNA in 12 of 14 samples. When the PBL 8 plus PBL 350 primers were used, only 1 of the tested 14 samples was positive, and the PL 1 plus PL 2 primers failed to amplify DNA from honey. Based on these results, the AF 6 plus AF 7 primers were used to analyze the other honey samples, as well as selected pollen, wax, and brood samples. Only the honey, pollen, wax, and brood samples from which *P. larvae* had been isolated were analyzed. The number of bacteria in the samples was classified according to the number of suspected *P. larvae* colonies on Columbia sheep blood agar plates. The isolates were identified and characterized by BioLog GP MicroPlate test panels. The evaluation of

TABLE 3. Comparison of different primer pairs used in the PCR assays on randomly selected honey samples

Sample	Code	Amplification with primer pair:			
		AF 1 + AF 2	AF 6 + AF 7	PBL 8 + PBL 350	PL 1 + PL 2
2	2001/37	–	+	–	–
4	2001/87	+	+	+	–
5	2001/983	–	+	–	–
6	2000/1019	+	+	–	–
7	2000/1020	+	+	–	–
11	AZ2000/692	–	–	–	–
12	AZ2000/691	–	+	–	–
13	AZ2000/497	+	+	–	–
14	AZ2000/1112	+	+	–	–
16	AZ95/5	–	+	–	–
17	AZ95/22	+	+	–	–
18	AZ95/4	–	–	–	–
19	BP10/2001	+	+	–	–
21	BP28/2001	+	+	–	–

the tests resulted in a probability value for the isolates being *P. larvae* subsp. *larvae*. The results of the PCR amplifications, isolations and BioLog tests are shown comparatively in Table 4. Altogether, 23 honey samples containing *P. larvae* subsp. *larvae* were tested by PCR, and 18 of them were found to be positive. The PCR-negative samples contained 3.2 to 33.8 CFU of bacteria by isolation, and the identification probability varied between 75 and 100%. The selected pollen, wax, and brood

samples were positive by PCR and showed a high BioLog probability value.

Another three honey samples (samples 24, 25, and 26), which did not contain *P. larvae* but from which several other *Bacillus* species had been isolated, were included in the study. These samples were clearly negative by PCR.

Five PCR products were sequenced, and the sequences were subjected to BLAST search to identify related sequences deposited in the GenBank database. The sequences uniformly exhibited the highest (100%) identity to the *P. larvae* subsp. *larvae* 16S rRNA gene partial sequence (accession no. AY030079). The sequence of the cultivated *P. alvei* nucleic acid showed only 93.13% identity to the *P. larvae* subsp. *larvae* sequence, and the highest (99.34%) identity was observed to the *P. alvei* strain DSM 29T partial 16S rRNA gene sequence (accession no. AJ320491). Because of the high identities of the nucleotide sequences of our strains to already available sequences, the sequences were not submitted to the GenBank database.

## DISCUSSION

The detection of contaminated honey plays an important role in the efficient control of American foulbrood. The small numbers of spores and the presence of other *Paenibacillus* and *Bacillus* species complicate the identification of *P. larvae* in honey samples. A large-scale screening of honey samples for *P.*

TABLE 4. Comparison of the efficacy of PCR, isolation, and BioLog identification techniques for the detection of *P. larvae* in bee products

Sample	Code	Type of sample	Country of origin	PCR results (AF 6 + AF 7)	Isolation (CFU/plate)	BioLog probability (%)
1	2001/36	Honey	Austria	+	>50	100
2	2001/37	Honey	Austria	–	3.55	98
3	2001/89	Honey	Austria	+	>50	100
4	2001/87	Honey	Austria	+	1.25	91
5	2001/983	Honey	Austria	–	33.8	100
6	2000/1019	Honey	Austria	+	0.8	100
7	2000/1020	Honey	Austria	+	1	91
8	B/768/92	Honey	Austria	+	1	100
9	AZ2000/696	Honey	Hungary	+	91	100
10	AZ2000/695	Honey	Austria	–	3.1	100
11	AZ2000/692	Honey	United Kingdom	–	5	100
12	AZ2000/691	Honey	France	+	>50	100
13	AZ2000/497	Honey	Saudi Arabia	+	10	99
14	AZ2000/1112	Honey	Turkey	+	>50	100
15	AZ98/76	Honey	USA	+	3.5	100
16	AZ95/5	Honey	Canada	+	3.35	100
17	AZ95/22	Honey	Romania	+	39	95
18	AZ95/4	Honey	Mexico	–	17	75
19	BP10/2001	Honey	Sardinia/Italy	+	1.58	100
20	AZ2001/94	Honey	Austria	+	6.2	99
21	BP28/2001	Honey	Austria	+	2	96
22	BP31/2001	Honey	Austria	+	>50	99
23	ÜW3	Honey	Austria	+	3.6	96
24	BP6/2000	Honey	Austria	–	0	0
25	AZ2001/280	Honey	India	–	0	0
26	AZ2001/325	Honey	India	–	0	0
27	ÜW5	Pollen	Germany	+	4.5	100
28	BP79/2000	Pollen	The Netherlands	+	0.25	100
29	W8	Wax	Austria	+	0.2	91
30	Wabe1	Brood	Austria	+	>50	100

larvae by classical isolation and identification methods is rather time-consuming, laborious, and expensive.

PCR is a quick and reliable method that is widely used in microbiological diagnostics for the detection of specific nucleic acid sequences in biological materials. In this study, we tested different PCR systems for the detection of *P. larvae*-specific DNA in honey and compared the sensitivity and specificity of the PCR assays with isolation and biochemical characterization methods.

To date, only two relatively short genomic regions of *P. larvae* have been sequenced and deposited in the GenBank database. Five primer pairs were selected from the 16S rRNA gene region, and three primer pairs were selected from the metalloproteinase precursor gene region (Table 1). All of them functioned properly on DNA extracts from *P. larvae* colonies.

The sensitivity of the PCR assays using different primers was determined by serial dilutions of *P. larvae* DNA isolated from bacterial colonies. The primer pair AF 6 plus AF 7 showed the highest sensitivity, amplifying *P. larvae* nucleic acid from as little as 0.05 CFU of cultured bacteria; primer pair AF 1 plus AF 2 exhibited a similarly high sensitivity (0.9 CFU). The primer pairs designed in the metalloproteinase precursor gene region showed significantly lower sensitivity ( $10^5$  to  $10^7$  CFU).

To assess the specificity of the primers used, the PCR assays were performed on other *Paenibacillus* and *Bacillus* species as well. Although the primers were selected from nucleotide sequences which are relatively specific for *P. larvae*, they also amplified, to a certain extent, DNA from other *Paenibacillus* and *Bacillus* species. The most sensitive primers for the detection of *P. larvae* (AF 6 plus AF 7; specific amplification as low as 0.05 CFU) also amplified *P. alvei* and *B. subtilis* strains at 2.6 and 11 CFU, respectively. The tested primer pair from the metalloproteinase gene region showed a rather low sensitivity for *P. larvae* ( $10^5$  CFU) but a relatively high specificity: the other tested bacteria reacted only at  $10^7$  CFU.

To investigate the practical value of the assays, honey samples were tested by PCR with the selected primers. Since bacterial spores are more resistant to proteinase K treatment than are vegetative cells, the routine DNA isolation methods have to be complemented by additional treatments to extract nucleic acid from spores. In this study, we compared the DNA isolation efficacy of three extraction methods. Two classical methods, using chemicals (guanidium thiocyanate and cetyltrimethylammonium bromide) for the lysis of the spores followed by phenol-chloroform extraction of the DNA, and one enzymatic (lysozyme) treatment followed by proteinase K digestion were tested. The methods were equally efficient (seven successful isolations from eight *P. larvae*-containing honey samples); however, with the different methods, different samples were negative. Since the classical methods are rather time-consuming, need special facilities, and result in dangerous waste material, lysozyme treatment followed by proteinase K digestion was chosen for further applications. Also, this DNA extraction method resulted, following PCR, in clear electrophoretic bands with larger amounts of amplified DNA compared to the other methods.

The PCR assays were applied to honey, pollen, wax, and brood samples. Practically only the AF 1 plus AF 2 and AF 6 plus AF 7 primers could detect *Paenibacillus*-specific nucleotide sequences in these samples (Table 3). The primers AF 6

plus AF 7 showed the highest sensitivity, detecting *P. larvae* in 18 of 23 contaminated honey samples (78.3%). This primer pair also detected *P. larvae* from pollen, wax, and brood samples. With honey samples, this PCR technique was specific for *P. larvae*, because it did not amplify DNA from honey samples which contained saprophytic *Bacillus* species but no *P. larvae* (Table 4). The determined nucleotide sequences of selected PCR products also support the specificity of this assay. Although the isolation and biochemical characterization of *P. larvae* from honey samples were found to be more sensitive and specific than PCR, PCR should still be considered for large-scale screening of American foulbrood in honey samples because of its rapidity and moderate expense.

Govan et al. also described a PCR detection method for rapid identification of *P. larvae* (8). Their system was developed for the identification of *P. larvae* cultivated from honey samples in semiselective medium (and not for direct detection in honey). We included their published primers in our studies (PL 1 plus PL 2 primers) for comparison. These primers detected *P. larvae* nucleic acid at a level of  $10^4$  CFU and failed to detect *P. larvae* DNA in honey samples.

The sensitivity of the PCR assays could be further improved by carrying out a second, nested amplification step. Certain combinations of our primers would allow a second-round PCR. On the other hand, the risk of cross-contamination, a lower specificity, a longer duration, and enhanced costs reduce the usability of nested PCR in large-scale screenings of honey samples.

To summarize, in this study we investigated quite intensively the applicability of PCR for the detection of *P. larvae*, the causative agent of American foulbrood, and found certain PCR assays to be appropriate for quick screening of honey samples for the presence of *P. larvae*; the results, however, should be confirmed by isolation and biochemical characterization of the bacteria. The specificity of the PCR assays could be improved by the use of primer sequences from other genomic regions, which are more specific for *P. larvae* than are the currently available sequences. For this approach, however, large genome segments of *P. larvae* and related bacteria have to be sequenced beforehand.

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