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Bacteriology

A novel multiplex PCR assay to detect and distinguish between different types of *Paenibacillus larvae* and *Melissococcus plutonius*, and a survey of foulbrood pathogen contamination in Japanese honey

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ABSTRACT. *Paenibacillus larvae* and *Melissococcus plutonius* are the causative agents of American and European foulbroods of honey bees, respectively. Since their virulence and resistance to disinfectants differ depending on the genotypes/phenotypes of the strains, the discrimination of strain types is important for the effective control of these diseases. Methods to detect and differentiate pathogens in honey are useful for surveying the contamination status of beehives/apiaries. In the present study, we selected a sequence (GenBank accession no. FI763267) as the specific target for enterobacterial repetitive intergenic consensus (ERIC) II-type *P. larvae* strains for the first time and developed a novel multiplex PCR assay that precisely distinguishes between the major types of foulbrood pathogens (ERIC I and II *P. larvae* and typical and atypical *M. plutonius*) in one reaction. In addition, we found that commercially available kits designed for DNA extraction from *Mycobacterium* in feces efficiently extracted DNA from foulbrood pathogens in honey. Using the multiplex PCR assay and DNA extraction kits, all the targeted types of *P. larvae* and *M. plutonius* were detected in honey spiked with the pathogens at a concentration of 100 bacterial cells/strain/ml. Moreover, 94% of the Japanese honey samples examined in the present study were contaminated with one or more types of the foulbrood pathogens. These results indicate that the newly developed methods are useful for detecting foulbrood pathogens in honey. The epidemiological information obtained by these methods will contribute to the effective control of foulbroods in apiaries.

KEY WORDS: Japanese honey, *Melissococcus plutonius*, multiplex PCR, *Paenibacillus larvae*, typing

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The maintenance of healthy honey bee colonies, particularly *Apis mellifera*, is not only important for the production of honey, but also for a variety of agricultural and horticultural crops because bees are the most important commercial pollinators. The healthy development of larvae is of critical importance for the maintenance of healthy colonies because most larvae grow into worker bees and undertake various tasks, including the cleaning and construction of hives, rearing larvae, colony defense, and foraging. Therefore, diseases that affect bee larvae seriously threaten the maintenance of healthy colonies.

American foulbrood (AFB) and European foulbrood (EFB) caused by *Paenibacillus larvae* and *Melissococcus plutonius*, respectively, are major infectious diseases affecting bee larvae. Outbreaks of these diseases may cause colony collapse and result in economic losses in the agricultural industry. Both pathogens are Gram-positive bacteria that orally infect larvae via brood food,

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

such as royal and worker jellies, pollen, and honey. *P. larvae* strains are classified into five enterobacterial repetitive intergenic consensus (ERIC) types (ERIC I–V) by repetitive-element PCR [9, 24] and 34 sequence types (STs) by multilocus sequence typing (MLST) (as of January 2022, https://pubmlst.org/plarvae/). *M. plutonius* strains have been grouped into three clonal complexes (CC3, CC12, and CC13) by MLST (as of January 2022, https://pubmlst.org/mplutonius/), and CC3 and CC13 strains with fastidious cultural characteristics and CC12 strains with non-fastidious characteristics are also referred to as typical and atypical strains, respectively [4, 10, 14, 30, 55]. In Japan, ERIC I and II are the only genotypes found in AFB cases, and the isolation of ERIC II *P. larvae* has recently been increasing [57]. While typical (CC3 and CC13) and atypical (CC12) *M. plutonius* have both been detected in Japanese cases of EFB, the isolation of the latter has been more frequent [4, 55].

P. larvae is a spore-forming bacterium. Dead larvae and dried larval scales in diseased colonies contain a large number of its endospores. These larval remains contribute to disease transmission within and between colonies [5, 36, 53]. *P. larvae* spores exist not only in diseased colonies, but also in clinically healthy colonies. Honey in beehives may be contaminated by *P. larvae* spores 2–3 years before the detection of clinical signs [58]. Although *M. plutonius* is not a spore-forming bacterium, it is also found in honey [18, 32]. Therefore, honey is a useful material for surveying the contamination of beehives/apiaries by pathogens.

As the detection and isolation of foulbrood pathogens from honey using culture methods is time consuming and not highly sensitive [13, 18, 25, 32, 40], the direct extraction of bacterial DNA from honey and the subsequent detection of specific genes using PCR techniques are useful as rapid and highly sensitive detection methods for foulbrood pathogens. In honey, *P. larvae* exists as endospores that resist a wide variety of physical and chemical stresses, while the non-spore-forming bacterium *M. plutonius* is present in honey as vegetative cells. Several DNA extraction methods for *P. larvae* spores in honey have been reported to date [1, 7, 33, 37, 44, 50], and honey has also been used as a material for the extraction of *M. plutonius* DNA [38]. However, to the best of our knowledge, there are currently no optimized methods to simultaneously extract DNA from *P. larvae* spores and *M. plutonius* vegetative cells in one tube. Virulence and resistance to disinfectants by both *P. larvae* and *M. plutonius* vary depending on strains [9, 10, 23, 24, 28, 35, 39, 42]. For example, *P. larvae* ERIC II strains are known to be more virulent for the individual larva [9, 23, 24] and more resistant to chlorine-based disinfectants [42] than ERIC I strains. Similarly, in *M. plutonius*, atypical strains have higher toxicity against larvae [39] and higher resistance to chlorine-based disinfectants [42] than typical strains. Therefore, differentiating between the genotypes/phenotypes of pathogens is also important for the accumulation of epidemiological information, understanding the ecology of pathogens, and planning comprehensive control measures based on characteristics of the pathogens present in apiaries. However, PCR methods to detect and differentiate between the major types of *P. larvae* and *M. plutonius* in one reaction have not yet been reported.

Therefore, in the present study, we constructed a novel multiplex PCR that enables the detection and differentiation of ERIC I and II *P. larvae* and typical and atypical *M. plutonius* in one reaction. Moreover, we selected commercially available kits that efficiently and simultaneously extract DNA from *P. larvae* spores and *M. plutonius* vegetative cells. Furthermore, using the developed techniques, we investigated the foulbrood pathogen contamination status of Japanese honey.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in the present study are listed in Table 1 and Supplementary Table 1. As foulbrood pathogens, 15 *P. larvae* strains (9 ERIC I and 6 ERIC II strains) and 18 *M. plutonius* strains (9 typical and 9 atypical strains) isolated in Japan were selected in order to cover all STs found in AFB and EFB cases in Japan. ATCC 35311, the type strain of *M. plutonius* purchased from the American Type Culture Collection (Manassas, VA, USA) was also included. The representative strains of *P. larvae* and *M. plutonius* listed in Table 1 were used in sensitivity tests of the newly developed multiplex PCR assay. Sixty-five other bacterial strains, including those isolated from Japanese honey [43] and honey bees [3], were used in addition to the foulbrood pathogens for specificity tests. Strains ATCC 6344 and ATCC 64, the type strains of *Paenibacillus alvei* and *Brevibacillus laterosporus*, respectively, were purchased from the American Type Culture Collection (Supplementary Table 1). The four strains listed in Table 1 were also employed as positive controls for the tests.

Primers and conditions for the multiplex PCR assay

Primers for the multiplex PCR assay are listed in Table 2. The multiplex PCR assay was performed using the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany) in a final reaction volume of 20 µl containing 10 µl of 2 × QIAGEN Multiplex PCR Master Mix, 2 µl of Q-solution, an appropriate concentration of each primer (Table 2), and 1 µl (for the sensitivity test)

Table 1. Strains used as positive controls for the multiplex PCR assay

Strain	Bacterial species	Genotype[a]	Source	References	Description
DTK386	*Paenibacillus larvae*	ST15, ERIC I	Diseased brood	[31, 57]	Strain I in reference [31]
DTK384		ST10, ERIC II	Diseased brood		Strain N in reference [31]
DAT606	*Melissococcus plutonius*	ST3, CC3	Diseased larvae	[4]	A typical strain
DAT561		ST12, CC12	Diseased larvae		An atypical strain

a) ST, sequence type; ERIC, enterobacterial repetitive intergenic consensus; CC, clonal complex.

Table 2. PCR primers used in the present study

Target			Primer				
Bacterial species	Genotype/ phenotype[a]	Gene/sequence [reference] (accession number)	Primer name	Sequence (5′-3′)	Optimal final concentration for the multiplex PCR assay (μM)	Reference	PCR product size (bp)
Paenibacillus larvae	ERIC I–V	16S rRNA gene [13, 26] (X60619)	Plarvae1	AAGTCGAGCGGACCTTGTGTTTC	0.1	[13, 26]	973[b]
			Plarvae2	TCTATCTCAAAACCGGTCAGAGG	0.1	[13, 26]	
	ERIC I	Toxin 1 gene, *plx1* [8, 19] (KC456421)	Pl-I-F1	GTAGCAGGGGTTTCTTCAGATGACAATG	0.2	The present study	554
			Pl-I-R1	AAAGCTACATGGGGATTACTCACACTCG	0.2	The present study	
	ERIC II	The sequence reported as neutral invertase-like protein gene [20] (FI763267)	Pl-II-F1	ACGGTCTCCCGTGTTACACTGACGTATG	0.2	The present study	333
			Pl-II-R1	GTTGCAAACTTCGCGATGATGATC	0.2	The present study	
Melissococcus plutonius	Atypical	Fur family transcriptional regulator gene [3] (AP012282; locus tag, MPD5_0863)	Mp-A-F1	TCCAACGGCAGATGAAATCTATCGAGCC	0.2	The present study	257
			Mp-A-R1	ACCCTGTTAACTTACTTACAACGCCTTC	0.2	The present study	
	Typical	Na^+/H^+ antiporter gene, *napA* [3] (AP012200; locus tag, MPTP_0420–0421)	Mp-T-F	TGGTAGCTTAGGCGGAAAAC	0.2	[3]	187
			Mp-T-R	TGGAGCGATTAGAGTCGTTAGA	0.2	[3]	

a) ERIC, enterobacterial repetitive intergenic consensus. b) According to sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/), a 972- or 974-bp product may be amplified from some *P. larvae* strains.

or 2 μl (for the other tests) of template DNA. Cycling conditions consisted of an initial denaturation step at 98°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 90 sec and extension at 72°C for 60 sec, and a final extension step at 72°C for 10 min. All PCR amplifications were performed in T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Six microliters of the amplification product was electrophoresed (100 V, 30 min) through a 2% agarose gel and visualized by staining the gel with ethidium bromide.

Bacterial DNA extraction

To assess the sensitivity of the multiplex PCR assay, genomic DNA was manually extracted from representative *P. larvae* strains cultured on MYPGP agar [13] at 35°C for two days under air plus 5% CO_2 conditions and *M. plutonius* strains cultured on KSBHI agar [4] at 35°C for three days under anaerobic conditions according to the methods described by Okamoto *et al*. [43] and Arai *et al*. [4], respectively. In brief, bacterial cells cultured as described above were suspended in TE (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA [pH 8.0]) or 5 × TE, treated with lysozyme and mutanolysin and lysed by 10% sodium dodecyl sulfate. The lysates were extracted with an equal volume of phenol, phenol-chloroform-isoamyl alcohol (25:24:1) (PCI) and chloroform at least once, three times and once, respectively. Nucleic acids were then precipitated by ethanol and further treated with RNase and/or proteinase K. After additional extraction with PCI and chloroform, DNA was precipitated and rinsed by ethanol and dissolved in Tris-based buffer. Genomic DNA was serially diluted to a final concentration of 10 fg/μl and used in sensitivity tests.

Regarding specificity tests, genomic DNA was extracted from a broad range of bacterial species (Supplementary Table 1) grown on appropriate culture media using InstaGene Matrix (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. Genomic DNA was diluted to a final concentration of 0.25 ng/μl and used in the multiplex PCR assay.

Preparation of honey spiked with P. larvae *spores and* M. plutonius *cells*

Spore suspensions of *P. larvae* DTK386 (ERIC I) and DTK384 (ERIC II) were prepared according to previously described procedures [42], and spores in sterile water were stored at 4°C until used. The number of spores in a suspension was calculated using a hemocytometer under an inverted microscope (CKX41, OLYMPUS, Tokyo, Japan). To calculate the germination rates of spore suspensions, germinable spore concentrations were also measured by plating serial dilutions of spore suspensions onto MYPGP agar after a heat shock treatment at 80, 85, 90, 95, and 100°C for 10 min and counting colonies after the incubation of plates at 35°C for six days under air plus 5% CO_2 conditions.

M. plutonius typical strain DAT606 and atypical strain DAT561 cultured at 35°C for three days on KSBHI agar under anaerobic conditions were collected using sterile cotton swabs, suspended in KSBHI broth containing 10% glycerol, and kept at −80°C until used. Colony-forming units (CFU) per milliliter of *M. plutonius* cell suspensions were calculated by plating serial dilutions of suspensions onto Basal agar plates [18] and counting colonies after the incubation of plates at 35°C for three days under anaerobic conditions. Since *M. plutonius* cells develop in chains [54], we also calculated the average number of cells in a single chain in the suspensions after staining *M. plutonius* cells using Gram's method and counting cells in 100 chains under a microscope (CME, Leica, Wetzlar, Germany). A single colony on an agar plate originates from a single chain of cells; therefore, *M. plutonius* cell

concentrations (cells/ml) in suspensions were measured using the following formula: cells/ml=CFU/ml × the average number of cells in a single chain.

To prepare foulbrood pathogen-spiked honey, we selected honey harvested in New Zealand in which the occurrence of EFB has not been reported. The absence of *P. larvae* in honey was confirmed by plating sediments of honey onto MYPGP agar containing 20 µg/ml nalidixic acid and 10 µg/ml pipemidic acid [13] after a heat shock treatment at 80, 85, 90, 95, and 100°C for 10 min and incubating the plates at 35°C for six days under air plus 5% CO_2 conditions. The absence of foulbrood pathogens was also confirmed using the DNA extraction and multiplex PCR methods developed in the present study. Foulbrood pathogen-free honey was then spiked with appropriate amounts of DAT606, DAT561, DTK386, and DTK384 cell/spore suspensions to yield final concentrations of 1,000, 100, 10, and 1 bacterial cell(s)/strain/ml of honey.

Extraction of bacterial DNA from honey

We used four commercially available DNA extraction kits to extract bacterial DNA from honey: the DNeasy Plant Mini Kit (QIAGEN), which is recommended for the extraction of *M. plutonius* DNA from honey in the "Standard methods for European foulbrood research [18]", the DNeasy PowerSoil Kit (QIAGEN), which is designed to isolate DNA including that of bacterial spores from environmental samples, such as soil, and Johne-Spin ver. 2 and Johne Pure Spin (FASMAC Co., Ltd., Atsugi, Japan), which are the kits used to extract DNA from *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces in Japan. Five milliliters of honey was diluted to 50% (v/v) with sterile water and centrifuged at approximately 12,000 × *g* for 15 min to obtain sediments. After sediments were suspended in 500 µl of sterile water, bacterial DNA was extracted from suspensions using the four kits according to each manufacturers' instructions. In extraction by the DNeasy PowerSoil Kit, bacteria in sediments were mechanically lysed in PowerBead tubes by vortexing at the maximum speed for 10 min using the Vortex-Genie 2 mixer (Scientific Industries, Inc., Bohemia, NY, USA) and the vortex adapter 13000-V1-24 (QIAGEN). When DNA was extracted by Johne-Spin ver. 2 and Johne Pure Spin, bacteria in sediments were mechanically lysed in Beads tubes for the kits by vortexing at 4,000 rpm for 5 and 2 min, respectively, using the Multi-beads shocker MB3000 (Yasui Kikai Corp., Osaka, Japan).

Survey of foulbrood pathogen contamination in Japanese honey

To survey the contamination of Japanese honey by foulbrood pathogens, 116 Japanese honey samples were collected (Supplementary Table 2). Except for honey no. J96, which was kindly provided by an apiary, samples were commercially available honey with different origins (i.e., different apiaries, producers, and/or floral origins) and purchased between 2017 and 2020. Three honey samples (J12, J24, and J42) were produced by *Apis cerana japonica*, and the others by *A. mellifera*. All samples were stored at room temperature until used. To extract DNA, sediments were obtained from five milliliters of each honey sample, as described above, and bacterial DNA was extracted from the sediments using Johne Pure Spin and then investigated using the multiplex PCR assay developed in the present study. To confirm specific detection of the target genes from honey, five honey samples (honey nos. J67, J88, J97, J106 and J117), which showed positive results for all the targets, were randomly selected as representatives. Each target gene was reamplified individually from the selected samples under the same PCR conditions, directly sequenced as described previously [41] and analyzed by the BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Target genes and primers for the multiplex PCR assay

In the present study, we designed a novel multiplex PCR assay with five primer sets to detect and differentiate between the ERIC I, ERIC II, and the other ERIC-type strains of *P. larvae* and typical and atypical strains of *M. plutonius* in one reaction. To detect typical *M. plutonius*, we employed previously reported primers targeting the Na^+/H^+ antiporter gene (*napA*) [3]. To detect ERIC I *P. larvae* and atypical *M. plutonius*, we selected the toxin 1 gene (*plx1*) (DDBJ/EMBL/GenBank accession no. KC456421) and the Fur family transcriptional regulator gene (AP012282; locus tag, MPD5_0863), respectively, as targets based on previous studies [3, 8, 19] and designed new primer sets to give specific PCR products of easily distinguishable sizes. To select the target of ERIC II *P. larvae*, we investigated nine sequences reported as ERIC II specific in the previous study [20] by the BLAST search (http://blast.ncbi.nlm.nih.gov) and Sequencher ver. 5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA) using *P. larvae* genome sequence data (GenBank accession nos. NZ_CP019659, NZ_CP019687, NZ_CP019651, NZ_CP019717, NZ_CP019655, NC_023134, NZ_CP019652, NZ_CP020557, NZ_CP019794, NZ_CP020327). As the sequence deposited in the database as neutral invertase-like protein gene (accession no. FI763267) was identified as the sole ERIC II-specific sequence among the nine, we employed a specific primer set for the sequence for our multiplex PCR assay. To detect *P. larvae* genotypes other than ERIC I and II, we additionally employed previously reported *P. larvae*-specific primers targeting the 16S rRNA gene [13, 26]. The lengths of the primers ranged between 20 and 28 bp, and primer sequences and product sizes are listed in Table 2. The specificities of the primers against the DNA sequences of bacteria available in the GenBank database were assessed by BLAST searches.

Specificity and sensitivity of the multiplex PCR assay

To evaluate the specificity of the PCR assay developed in the present study, we employed a wide range of bacterial strains, including those isolated from honey and healthy and diseased honeybee larvae (Supplementary Table 1). Under the optimized conditions described in the Materials and Methods section, multiplex PCR primers yielded specific PCR products of the expected sizes from all *P. larvae* ERIC I (973 bp and 554 bp) and ERIC II (973 bp and 333 bp) strains and all *M. plutonius* typical (187 bp)

and atypical (257 bp) strains, while no products were generated from any other bacterial strains tested in the present study (Fig. 1 and Supplementary Table 1). Differently sized specific products were easily distinguishable from each other on agarose gels (Fig. 1). The multiplex PCR assay detected the 16S rRNA gene of *P. larvae* from 100 fg of genomic DNA, and the other genes were detected from 1 pg of genomic DNA (Fig. 2).

Comparison of commercial DNA extraction kits

P. larvae exists as endospores in honey, while *M. plutonius* is present as vegetative cells. To extract DNA from exceptionally tough spores, bead-based mechanical lysis methods using high-speed homogenizers are usually used. However, mechanical lysis methods may be too strong for vegetative cells and may damage their DNA, resulting in PCR failure. To employ efficient and optimal DNA extraction methods from honey that may be used for both the spore and vegetative forms of bacteria, we spiked the spores of ERIC I and II *P. larvae* and the vegetative cells of typical and atypical *M. plutonius* (Table 1) into foulbrood pathogen-

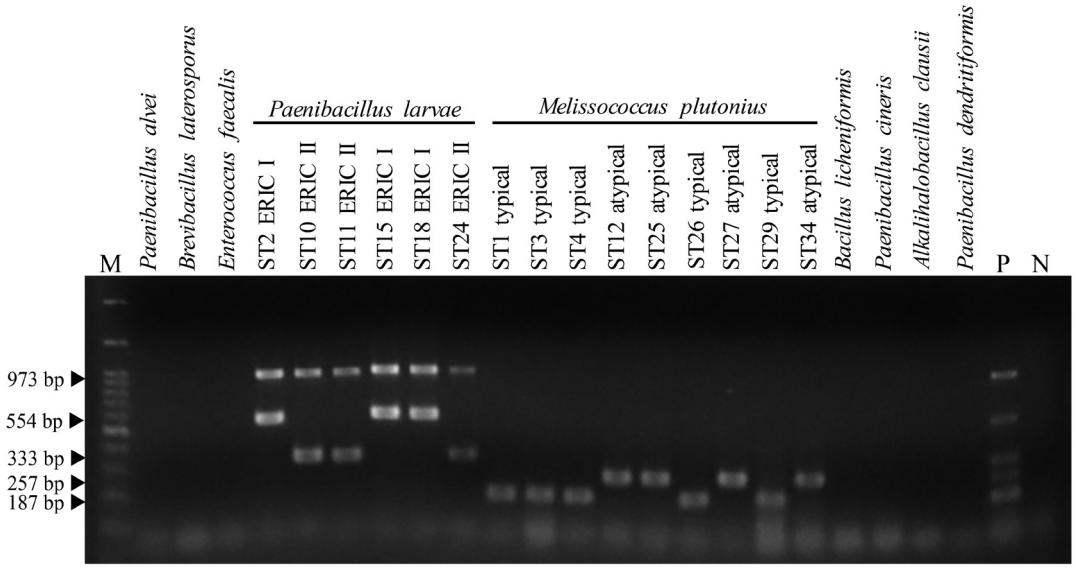

Fig. 1. Representative results of the specificity test of the multiplex PCR assay developed in the present study. DNA samples extracted from bacterial cultures were used for PCR. Bacterial species are indicated above each lane. ST, sequence type. ERIC, enterobacterial repetitive intergenic consensus. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *Paenibacillus larvae* DTK386 and DTK384 and *Melissococcus plutonius* DAT606 and DAT561). N, no template control.

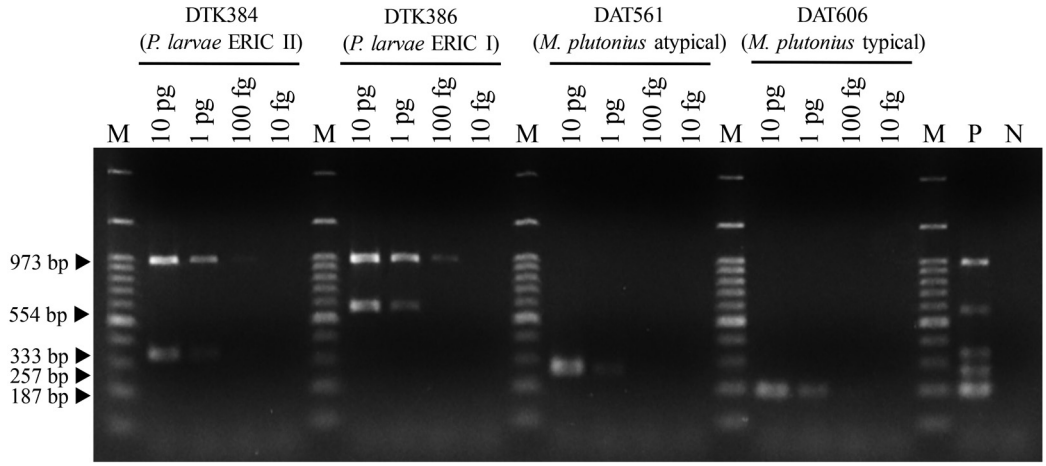

Fig. 2. Sensitivity of the multiplex PCR assay developed in the present study. Serial dilutions of DNA extracted from *Paenibacillus larvae* DTK384 (enterobacterial repetitive intergenic consensus [ERIC] II), *P. larvae* DTK386 (ERIC I), *Melissococcus plutonius* DAT561 (atypical), and *M. plutonius* DAT606 (typical) were used to investigate the sensitivity of PCR. The amount of DNA used as the template for each reaction is indicated above each lane. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *P. larvae* DTK386 and DTK384 and *M. plutonius* DAT606 and DAT561). N, no template control.

free honey at a concentration of 1,000 cells/strain/ml, extracted DNA from 5 ml of spiked honey using four commercial DNA extraction kits (DNeasy Plant Mini Kit, DNeasy PowerSoil Kit, Johne-Spin ver. 2 and Johne Pure Spin), and evaluated their DNA extraction efficiencies using the developed multiplex PCR assay. Among the four kits, DNeasy PowerSoil Kit, Johne-Spin ver. 2, and Johne Pure Spin adopt mechanical lysis methods using beads.

As shown in Fig. 3, *P. larvae* and *M. plutonius* DNA were not efficiently extracted from honey by the DNeasy PowerSoil Kit or DNeasy Plant Mini Kit. Although the 16S rRNA gene of *P. larvae* and the typical *M. plutonius*-specific gene were barely detected in the DNA samples extracted by the two kits, the other products were invisible on agarose gels (Fig. 3). In contrast, all target genes were detected from DNA extracted by Johne-Spin ver. 2 and Johne Pure Spin (Fig. 3). All genes were detectable when Johne Pure Spin was used, even those in honey spiked at a concentration of 100 bacterial cells/strain/ml (Fig. 4); therefore, we used Johne Pure Spin in subsequent experiments.

Since most of the target genes were undetectable in honey spiked at concentrations of 10 and 1 bacterial cell(s)/strain/ml (Fig. 4), the detection limit of this protocol (i.e., DNA extraction by Johne Pure Spin and the multiplex PCR assay) was 100 bacterial cells/strain/ml of honey. In the present study, DNA extracted from 5 ml of spiked honey was eluted with 50 µl of elution buffer, and 2 µl of the extract was used for one reaction of the multiplex PCR assay; therefore, any type of *P. larvae* and *M. plutonius* may be detected by the multiplex PCR assay when DNA from 20 cells is present in one PCR reaction.

Foulbrood pathogen contamination rates in Japanese honey

Using the developed multiplex PCR assay and DNA extracted from honey by Johne Pure Spin, we investigated the contamination rates of *P. larvae* and *M. plutonius* in Japanese honey using 116 honey samples (Supplementary Table 2). *P. larvae* and/or *M. plutonius* were detected in 94% of honey samples (Fig. 5A). Both pathogens were found together in 75% of samples,

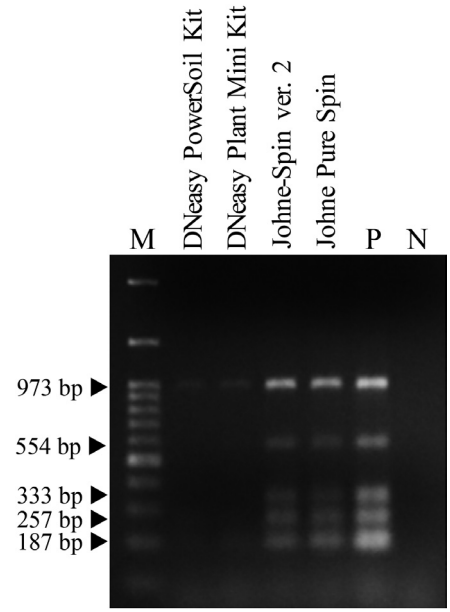

Fig. 3. Comparison of DNA extraction kits. Foulbrood pathogen-free honey was spiked with the *Paenibacillus larvae* spores of enterobacterial repetitive intergenic consensus (ERIC) I and II types (DTK386 and DTK384, respectively) and typical and atypical *Melissococcus plutonius* strains (DAT606 and DAT561, respectively) at a final concentration of 1,000 cells/strain/ml of honey. DNA samples were extracted from sediments from 5 ml of the spiked honey using four different commercial kits (DNeasy PowerSoil Kit [QIAGEN, Hilden, Germany], DNeasy Plant Mini Kit [QIAGEN], Johne-Spin ver. 2 [FASMAC Co., Ltd., Atsugi, Japan], and Johne Pure Spin [FASMAC]) according to each of the manufacturer's instruction, and 2 µl of each DNA sample was used as a template for each reaction in the multiplex PCR assay. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *P. larvae* DTK386 and DTK384 and *M. plutonius* DAT606 and DAT561). N, no template control.

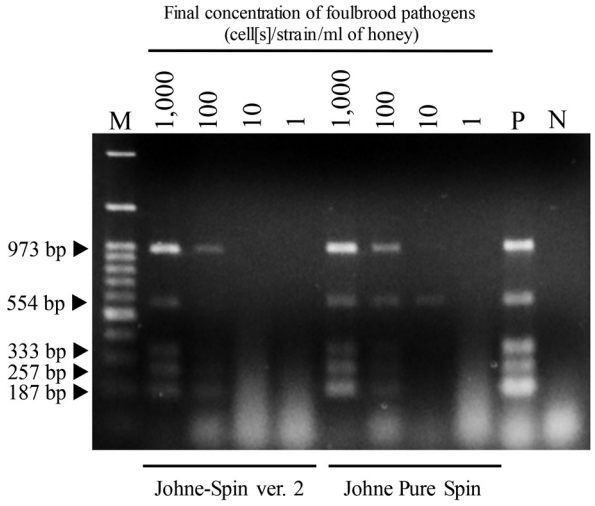

Fig. 4. DNA extraction efficiency from foulbrood pathogens in honey by Johne-Spin ver. 2 (FASMAC Co., Ltd., Atsugi, Japan) and Johne Pure Spin (FASMAC). Foulbrood pathogen-free honey was spiked with the *Paenibacillus larvae* spores of enterobacterial repetitive intergenic consensus (ERIC) I and II types (DTK386 and DTK384, respectively) and typical and atypical *Melissococcus plutonius* strains (DAT606 and DAT561, respectively). DNA samples were extracted from sediments from 5 ml of the spiked honey using the two kits, and 2 µl of each DNA sample was used as a template in each reaction of the multiplex PCR assay. Six microliters of the PCR product was run on a 2% agarose gel and stained with ethidium bromide. The final concentrations of bacterial cells in the spiked honey (cell[s]/strain/ml of honey) are indicated above each lane. M, molecular size marker (100-bp DNA ladder). P, positive control (a mixture of 0.5 ng of each DNA from *P. larvae* DTK386 and DTK384 and *M. plutonius* DAT606 and DAT561). N, no template control.

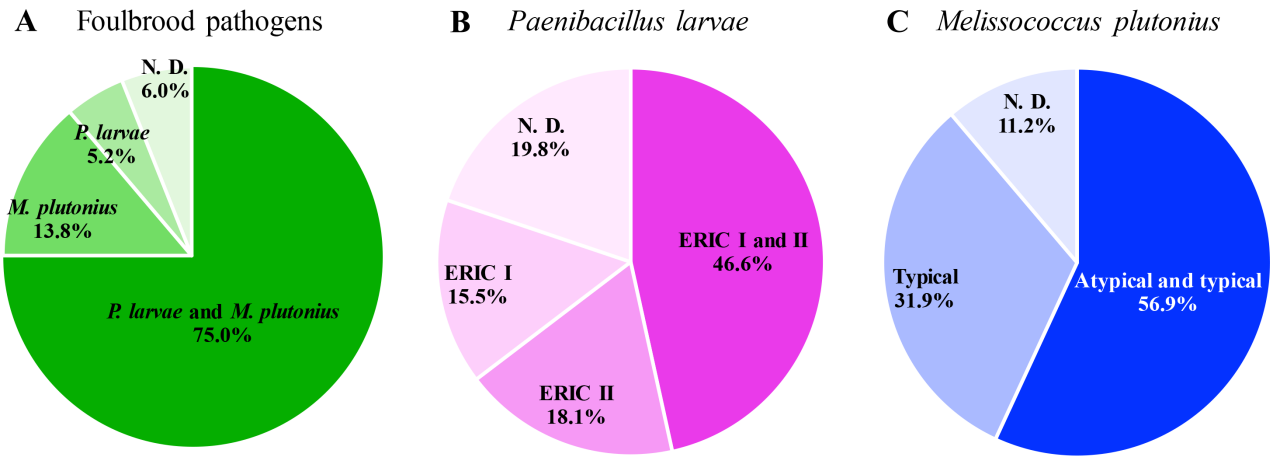

Fig. 5. Detection rates of (**A**) *Paenibacillus larvae* and *Melissococcus plutonius*, (**B**) enterobacterial repetitive intergenic consensus (ERIC) I and II types of *P. larvae*, and (**C**) typical and atypical *M. plutonius* from Japanese honey. DNA samples were extracted from the sediments of 5 ml of honey by Johne Pure Spin (FASMAC Co., Ltd., Atsugi, Japan), and 2 µl of each DNA sample was used as a template in each reaction of the multiplex PCR assay. N. D., not detected.

and 34.5% of samples were positive for all four targets (i.e., ERIC I and II *P. larvae* and typical and atypical *M. plutonius*) (Fig. 5A and Supplementary Table 2). ERIC I and II *P. larvae* were simultaneously detected in 46.6% of samples, while ERIC I and II were solely detected in 15.5 and 18.1% of samples, respectively (Fig. 5B). Typical and atypical *M. plutonius* were simultaneously detected in 56.9% of the samples, and 31.9% of honey samples were positive for typical *M. plutonius* alone (Fig. 5C). Of note, all the PCR products analyzed by sequencing were confirmed to be specific products, further supporting the specificity of the multiplex PCR and reliability of the survey results.

DISCUSSION

The detection of foulbrood pathogens in clinically healthy colonies facilitates the control and prevention of diseases, and honey is one of the useful materials for assessing the contamination status of bee colonies. Although cultivation is a traditional and popular technique for detecting bacterial pathogens from materials, the detection of foulbrood pathogens from honey bee samples using this technique is time consuming due to the relatively slow growth of pathogens. In addition, since *P. larvae* and *M. plutonius* require different fastidious culture conditions, they cannot be simultaneously isolated on the same culture medium. Furthermore, other bacteria contaminating honey overgrow and interfere with the isolation of foulbrood pathogens on agar media. To avoid the common limitations associated with culture methods, molecular diagnostic techniques, such as PCR, have been developed and are replacing culture methods. PCR techniques may be easily replicated in laboratories using commercially available reagents and detect more than one target in one reaction using multiple specific primer sets; therefore, they are useful for simultaneously detecting multiple bacterial species without needing to consider the culture characteristics of each bacterium.

Many PCR assays have been developed to detect foulbrood pathogens. Regarding *M. plutonius*, specific PCR assays that detect the 16S rRNA gene [16, 27, 38] and a real-time PCR assay for the manganese-dependent superoxide dismutase gene (*sodA*) [49] have been reported. In addition, Arai *et al*. [3] developed duplex PCR to easily detect and differentiate between typical and atypical *M. plutonius* strains. Many conventional and real-time PCR assays that target the 16S rRNA gene have been developed for *P. larvae* [7, 17, 26, 29, 33, 37, 46, 50]. Moreover, Piccini *et al*. [44] and Alippi *et al*. [1] reported PCR assays for the specific detection of *P. larvae* subsp. *larvae* (i.e., ERIC I and II strains under the current taxonomic assignments of *P. larvae* [22, 24]), while Beims *et al*. [8] developed a triplex quantitative PCR assay that specifically detect ERIC I strains. However, PCR assays specific for ERIC II strains have not yet been developed. In the present study, we identified the sole ERIC II-specific sequence (accession no. FI763267) for our multiplex PCR assay; therefore, our PCR is the first PCR that specifically detects *P. larvae* ERIC II strains. The multiplex PCR and triplex real-time PCR assays described by Garrido-Bailón *et al*. [21] and Dainat *et al*. [12], respectively, simultaneously detect *P. larvae* and *M. plutonius* in one reaction; however, these assays cannot distinguish between the genotypes of *P. larvae* and phenotypes of *M. plutonius*. Therefore, our PCR assay is also the first PCR assay that simultaneously detects and differentiates between the major ERIC types of *P. larvae* and typical and atypical strains of *M. plutonius*. However, the number of strains examined in the present study was still limited. Since *P. larvae* and *M. plutonius* are prohibited for import in Japan, we mainly used strains isolated in Japan for the specificity test, and, thus, further studies are needed to verify the specificity of our multiplex PCR assay using *P. larvae* and *M. plutonius* strains isolated in other countries.

Among the primers employed for the multiplex PCR assay, those for the 16S rRNA gene of *P. larvae* showed higher sensitivity (detection limit, 100 fg of genomic DNA) than the other primer sets (detection limit, 1 pg of genomic DNA) (Fig. 2). This may be attributed to the copy numbers of the target genes because *P. larvae* strains have eight copies of the 16S rRNA gene in the

chromosome [15].

Johne-Spin ver. 2 and Johne Pure Spin were both developed for the efficient extraction of DNA from acid-fast bacteria, such as *M. avium* subsp. *paratuberculosis* in bovine feces. According to the manufacturer, Johne Pure Spin produces DNA with higher quality than Johne-Spin by removing more PCR inhibitors. Although we used Johne Pure Spin for the survey of foulbrood pathogen contamination in Japanese honey, the results obtained suggest that Johne-Spin ver. 2 is appropriate for the same purpose (Fig. 3). Since both kits are widely used in local livestock hygiene service centers in Japan for the examination of Johne's disease, the inspection of foulbrood pathogens using the same kits will be easy to perform by these centers.

Regarding the extraction of DNA from honey using Johne Pure Spin, the detection limit of *P. larvae* DTK384 and DTK386 spores by the multiplex PCR assay was 100 spores/ml of honey. The germination rates of DTK384 and DTK386 spore solutions used in the present study were 13.5 and 2.1%, respectively; therefore, their detection limits correspond to 13.5 and 2.1 CFU/ml of honey, respectively. The detection limits of *P. larvae* spores from honey were previously reported to be 283 spores of *P. larvae* subsp. *larvae*/g of honey (*P. larvae* subsp. *larvae* detection PCR developed by Alippi *et al*. [1]) and 9 CFU/ml of honey (nested PCR developed by Lauro *et al*. [33]). Although DNA extraction methods differed between previous studies and the present study, the detection limit of *P. larvae* spores from honey by our methods was superior or similar to those of previously reported methods. In contrast, the sensitivities of real-time PCR assays targeting the 16S rRNA gene of *P. larvae* reported by Martínez *et al*. [37] and Rossi *et al*. [50] were higher than that of our PCR assay. When the UltraClean Soil DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) with a bead beating procedure [37] and the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) [50] were used for DNA extraction from honey, these assays detected *P. larvae* from 2 spores/g of honey and 1 CFU/g of honey, respectively. However, since these methods cannot distinguish between the ERIC types of *P. larvae*, our method is useful for assessing the contamination status of beehives/apiaries by a pathogen.

Limited information is currently available on *M. plutonius* detection protocols from honey samples. McKee *et al*. [38] employed hemi-nested PCR described by Djordjevic *et al*. [16] and detected *M. plutonius* from honey. However, the detection limit was not investigated in that study. In the present study, we successfully detected both typical (DAT606) and atypical (DAT561) *M. plutonius* from honey samples with a detection limit of 100 cells/ml of honey (Fig. 4). Since the average numbers of cells in a single chain of strains DAT606 and DAT561 used for the preparation of *M. plutonius*-spiked honey were 5.26 and 3.43 cells, respectively, the detection limits of typical and atypical *M. plutonius* were equivalent to 19 and 29.2 CFU/ml of honey, respectively. Although there are currently no reported findings for comparison with our results, the present study implies the sufficiently high sensitivity of our methods; our methods detected *M. plutonius* in 88.8% of the Japanese honey samples tested (Fig. 5). On the other hand, Hornitzky and Smith [32] detected *M. plutonius* in only 6.2% of bulk honey samples in EFB endemic areas using their culture procedure. Since the honey samples used differed between the present and previous studies, the two detection rates cannot be directly compared. Nevertheless, the high detection rate in the present study suggests the usefulness of our DNA extraction and multiplex PCR methods for the investigation of *M. plutonius* contamination in honey.

In previous studies using bacterial culture methods, the contamination rates of *P. larvae* spores in honey collected from Italy, Uruguay and Poland were 35.6–56% [2, 6, 45, 47]. In a quantitative PCR assay, 59.2% of honey samples in Italy were revealed to be contaminated with *P. larvae* [11]. Ribani *et al*. [48] also reported the detection of *P. larvae* in 49 and 79% of honey samples collected from Italy and other countries (North, Central, and South American, Asian, African, and Oceanian and other European countries), respectively, using a conventional PCR assay. They also demonstrated that *M. plutonius* was positive in 87% of all analyzed samples and that both pathogens were co-amplified from 50% of samples. According to these findings, both pathogens contaminate honey with high probability. In our survey, the *M. plutonius*-positive rate (88.8%) in Japanese honey was similar to that reported by Ribani *et al*. [48], while the *P. larvae*-positive rate in the present study (80.2%) was higher than that in previous studies in other countries [2, 6, 11, 45, 47, 48]. As all the honey-derived PCR products sequenced in the present study were specific products, these positive rates strongly suggest the wide distribution of foulbrood pathogens in Japan. Since commercial honey is generally dispensed from bulk honey collected from more than one honey bee colony and/or farm with a different infection status, the infectious status of each colony was unknown in most cases in the present study. However, J91 honey was a comb honey sample harvested from a single colony and showed positive for all of the target pathogens (Supplementary Table 2), suggesting that a honey bee larva in a single colony may simultaneously encounter several pathogens.

In Japan, the number of apiaries including weekend beekeepers is approximately 10,000 houses. According to the annual statistics of foulbrood diseases conducted by the Ministry of Agriculture, Forestry and Fisheries in Japan, approximately 30–60 AFB/EFB cases were officially reported annually in recent years. Despite the high positive rate of foulbrood pathogens in Japanese honey, the number of reported foulbrood cases is relatively small, and this may be in part attributed to the disruption of diseased colonies before the diagnosis of foulbroods or the misdiagnosis of diseased colonies. Alternatively, the approved prophylactic for AFB (tylosin) may effectively control not only AFB, but also EFB. The growth of all Japanese *P. larvae* and *M. plutonius* isolates tested to date was inhibited by tylosin at low concentrations [56, 57]. The hygiene behavior of honey bees, i.e., the removal of an infected brood before the infectious stage of *P. larvae*, is the main mechanism underlying resistance to AFB [34, 51, 52]; therefore, the majority of honey bee colonies in Japan may be healthy and strong due to good beekeeping practices, which lead to efficient removal of infected brood by honey bees resulting in clinical healthy colonies without visible clinical signs regardless of the presence of foulbrood pathogens. Although further analyses are needed to identify the factors causing the relatively small number of reported foulbrood cases in Japan, countermeasures, such as the disinfection of beehives and the use of the prophylactic for AFB, will be required for prevention if pathogens are detected in honey. In contrast, if pathogens are not detected, the apiaries can be considered at a low risk of developing foulbroods in the year. In these apiaries, the amount of antibiotics can be reduced.

Although our honey investigation method was originally developed to survey the contamination status of beehives/apiaries by foulbrood pathogens, it will also contribute to the appropriate use of prophylactics in apiaries.

CONFLICT OF INTEREST. The authors declare no conflicts of interest associated with this manuscript.

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