Detection of macrolide resistance genes, ermC and ermB, in Japanese honey using real-time PCR assays

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ABSTRACT. American foulbrood (AFB) is a honeybee disease caused by Paenibacillus larvae, and tylosin is used as the prophylactic in Japan. Honey contains macrolide-resistant bacteria that are a potential source of genes that may confer tylosin resistance to P. larvae. To investigate the potential risk of such genes in Japanese honey, we developed real-time PCR assays for the detection of important macrolide resistance genes, ermC and ermB, and analyzed 116 Japanese honey samples with known contamination status of P. larvae. Consequently, 91.38% of samples contained ermC and/or ermB, and 71.55% of samples contained both ermC and P. larvae, suggesting the possible emergence of tylosin-resistant P. larvae in Japan. Therefore, judicious use of the prophylactic is essential in maintaining its effectiveness.

KEYWORDS: ermB, ermC, macrolide resistance gene, Paenibacillus larvae, real-time PCR
each suspension was calculated using hemocytometers under an inverted microscope (CKX41, OLYMPUS, Tokyo, Japan). The spore
et al (Supplementary Table 1). The number of spores in 
], with certain modifications to the culture conditions ( [8
according to Ohashi 
ermC-
ermB in honey samples using our assays, we prepared bacterial spore-spiked honey using 
and 
-free honey harvested in Thailand.
that carry plasmids containing 
genes, respectively [9]. To determine the detection limit of these representative bacteria 
ermB 
ermC 
amplicons and found that (71.7–75.2°C), we determined the sequences of all 
amplicons exhibited sequence variations (Fig. 
ermC 
ermC 
ermC 
ermC 
and 
ermB 
ermC genes are carried by a single or limited bacterial 
imply that 
2). The higher detection rate of 
is carried by a wide variety of 
ermC 
ermC 
ermC and the sequence variations in 
ermB 
amplicons to be 10 spores/mL honey. The melting point temperatures of the 
and 
amplicons were 75.0°C and 75.6°C, respectively.
Okamoto 
extracted from 5 mL of each spore-spiked honey sample using the Johne Pure Spin kit (FASMAC Co., Ltd., Atsugi, Japan), according
suspensions were then added to honey samples to obtain final solutions of 1,000, 100, 10, and 1 spore(s)/mL honey. Genomic DNA was 
extracted from 5 mL of each honey sample as previously described [10], with certain modifications to the culture conditions (Supplementary Table 1). The number of spores in each suspension was calculated using hemocytometers under an inverted microscope (CKX41, OLYMPUS, Tokyo, Japan). The spore suspensions were then added to honey samples to obtain final solutions of 1,000, 100, 10, and 1 spore(s)/mL honey. Genomic DNA was extracted from 5 mL of each spore-spiked honey sample using the Johne Pure Spin kit (FASMAC Co., Ltd., Atsugi, Japan), according to the manufacturer’s instructions. Positive controls and non-template negative controls were placed on each reaction plate. 

\[ \text{PCR conditions} \]

\[
\begin{align*}
\text{ermC} & \quad \text{erm}_{C,qF} \quad \text{CTGATAAGYGAGCTATTCAC} \\
& \quad \text{erm}_{C,qR} \quad \text{ATCGTGGAATACGGGTTTGC} \\
& \quad \text{PCR conditions} \quad 95°C \text{ min–94°C 15 sec, } 55°C \text{ 30 sec, 72°C 30 sec (45 cycles)}
\end{align*}
\]

\[
\begin{align*}
\text{ermB} & \quad \text{erm}_{B,qF} \quad \text{CCGCCATAACCACAGATGTTC} \\
& \quad \text{erm}_{B,qR} \quad \text{ACTTTGGCGTGTTTCATTGC} \\
& \quad \text{PCR conditions} \quad 95°C \text{ min–94°C 15 sec, } 55°C \text{ 30 sec, 72°C 30 sec (45 cycles)}
\end{align*}
\]

* Presence of the 
ermC gene was assessed at two different annealing temperatures during the PCR cycle. All PCR results from the two conditions were used to calculate the 
ermC detection rates.

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]

\[ \text{erm\textit{C}} \]

\[ \text{erm\textit{B}} \]
species cannot be excluded. As reported from a previous study of our research group [9], Japanese honey contains a wide variety of bacteria of different genus, such as Bacillus, Paenibacillus and Alkalihalobacillus. Among the bacterial isolates analyzed in the previous study, the ermA and ermB genes have only been identified in O. oncorhynchi subsp. incaldanensis and Paenibacillus sp., respectively [9]. However, information on other bacterial species in honey that carry erm genes remains unclear. Therefore, further investigation is essential to elucidate the bacterial species that carry macrolide resistance genes and their distribution in Japanese honey.

P. larvae becomes tylosin-resistant if it acquires the ermA gene [9]; therefore, the co-contamination of honey with ermA and P. larvae suggests the potential risk of the emergence of macrolide-resistant P. larvae in beehives and apiaries. To further investigate this risk, we retrieved the P. larvae-positive/negative data of the same 116 honey samples analyzed in an earlier study conducted by our research group [10] and compared it with the ermA-positive/negative data obtained from the present study. Surprisingly, both ermA and P. larvae were detected in 71.55% of honey samples (Fig. 1B, Supplementary Fig. 1, and Supplementary Table 2). Since most samples analyzed in this study were bulk honey, the detection rate did not represent the status of a single honey bee colony. In addition, as our assays do not provide insight into the transferability of ermA, the detected gene may not have the potential to transfer to P. larvae and subsequently confer tylosin resistance to the bacterium. However, in apiaries contaminated with both ermA and P. larvae, judicious use of tylosin is recommended to prevent the selection of macrolide-resistant P. larvae strains. Although we cannot deny the possibility that bacteria in honey may contain other antibiotic resistance genes that could confer macrolide resistance to P. larvae, tylosin can be efficiently used in the control of AFB with a low risk of tylosin-resistant P. larvae selection in the apiaries where only P. larvae is detected.

To the best of our knowledge, this is the first study that focuses on ermA genes in honey. The real-time PCR assays developed in this study can be applied in apicultural industries to ensure judicious use of antimicrobials. Future improvement of the assays for specific detection of transferable ermA/ermB genes would further help efficient and appropriate control of AFB.

CONFLICT OF INTEREST. The authors have no conflicts of interest to declare.

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Fig. 1. Detection rates of (A) macrolide resistance genes, ermA and ermB, and (B) Paenibacillus larvae and ermA from Japanese honey.

DNA was extracted from the sediments of 5 mL of honey using the Johne Pure Spin kit (FASMAC Co., Ltd., Atsugi, Japan), and 1 µL of the extracted DNA from each sample was used as a template in real-time PCR assay to detect ermA/ermB. The detection rate of P. larvae from the honey samples was retrieved from a previous study conducted by our research group [10]. N. D., not detected.

Fig. 2. Sequence variations of the ermA gene fragments detected from Japanese honey samples. The ermA gene sequence of Oceanobacillus oncorhynchi subsp. incaldanensis (strain J18TS1) was retrieved from the GenBank database (accession no. LC586988) and compared with the amplified gene sequences from Japanese honey. The ermA gene sequence of J18TS1 is shown at the bottom. The numbers indicate the position of nucleotides in the ermA gene of strain J18TS1. Nucleotide differences are shown with colored backgrounds. R=A or G; Y=C or T.
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