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Research Paper

Molecular Detection of *Enterococcus faecalis* as a Secondary Agent of European Foulbrood Disease in Honey Samples

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ABSTRACT

Introduction: European foulbrood (EFB) disease is the most important bee larvae disease in honey bees, caused by *Melissococcus plutonius*, and subsequently the larvae are infected with secondary bacteria such as *Enterococcus faecalis*, *Brevibacillus laterosporus*, and *Paenibacillus alvei*. Therefore, the presence of these secondary bacteria can be considered as a remarkable sign for EFB disease. Since the preparation of honey bee samples for the assessment of apiary health presents principal problems, this research aimed to use honey samples to track *E. faecalis* as a secondary agent of EFB disease in apiaries all over Iran.

Materials & Methods: A total of of 260 apiaries were selected, and honey samples were collected during the autumn and winter of 2023. After preparation of the honey samples according to the standard protocol, genomic DNA was extracted from the samples, and then a pair of specific primers was used to amplify the target fragment of *E. faecalis* using the polymerase chain reaction (PCR) technique. As the positive and negative controls, the standard bacterium and the distilled water were used, respectively. A sensitivity test was performed using specific concentrations of DNA extracted from the standard *E. faecalis* under the corresponding optimal PCR reaction conditions, and the appropriate concentration was considered for the PCR reaction of the collected samples.

Results: PCR results showed that 119(46%) of the 260 honey samples were positive for *E. faecalis*.

Conclusion: The findings show that the quick and easy use of honey samples for diagnosing *E. faecalis* can be introduced as an appropriate method to detect this bacterium. Therefore, honey samples can be recommended as a source for the detection of *E. faecalis*. Also, severe infection of the Iranian apiaries with *E. faecalis* as an indicator of EFB disease should be recognized as a very significant problem.

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1. Introduction

In addition to producing important products like honey, beeswax, royal jelly, and propolis, honey bees have a vital role in the pollination of plants [1] by increasing their diversity and ultimately improving crop productivity. Therefore, they have a key role in food security [2]. In recent years, however, a significant increase has occurred in mortality in managed honey bee colonies around the world [3]. Several agents such as viruses, fungi, and bacteria infect honey bees. European foulbrood (EFB) disease is caused by *Melissococcus plutonius*, an anaerobic gram-positive lanceolate bacterium [4, 5]. Brood diseases (EFB and American foulbrood [AFB]) are the most significant reasons for honey bee larval mortality and can cause the weakening and subsequent death of infected colonies [6]. An EFB-infected colony shows covered and uncovered cells that are irregularly and sporadically distributed on the brood frame. The most commonly observed symptom in larvae, depending on the severity of the disease, is a change in color from white to yellow, brown, and even greyish-black [7]. Following *M. plutonius*, secondary bacteria, for example, *Enterococcus faecalis*, *Paenibacillus alvei* and *Brevibacillus laterosporus*, may attack infected colonies [8]. It should be kept in mind that following an *M. plutonius* infection, contamination by the secondary invaders does not necessarily increase disease lethality. It may be just a colonization of weak or dead larvae and can be a remarkable sign for EFB disease [9].

Regarding the lack of treatments for pathogen-based honey bee diseases, management strategies may be the best way to control these diseases in apiaries. Therefore, detection and elimination of pathogenic agents in hives could help improve apiary health. Honey samples, as a resource of environmental DNA that are easily accessible, can be useful for the detection of the pathogens and honey bee epidemiological studies [10]. In this study, the presence of *E. faecalis* as a secondary agent of EFB disease was evaluated using polymerase chain reaction (PCR) in honey samples from apiaries throughout the country.

2. Materials and Methods

2.1. Data collection

Sampling was randomly accomplished based on the number of apiaries in Iran across provinces. According to the report by the Iran Veterinary Organization, the prevalence rate of EFB disease was considered to be 40%. Cochran's formula (Equation 1) was used for calculating the sample size [11]:

1. $N=z^2\ [pq]/d^2$

Where, d or error was considered to be 0.06 and p, q, and z (the standard normal variable) were equal to 0.4, 0.6 and 1.97, respectively. The confidence factor was equal to 95%. So, N≅260. Consequently, from 31 provinces, 260 apiaries were considered, while 5 mL of honey was collected from each apiary in sterile bottles. For sampling, in each apiary, a number of hives were randomly selected, and the clinical symptoms of the disease were ignored. Next, the honey samples from each apiary were pooled, and then all pooled honey samples from all provinces were sent to the lab to check for the presence of the bacterium under study.

2.2. Bacterial culture, sample preparation, DNA extraction, and PCR

According to Forsgren et al. [7], the standard bacterium (provided by the Razi Vaccine and Serum Research Institute, Iran) was cultured in a specific medium (blood agar) for the positive control. Honey samples were prepared by taking 5 mL of honey and heating it at 40 ˚C for 10 min. The samples were diluted 1:1 with PBS and centrifuged at 6000 g for 20 min. The supernatant was discarded, and the precipitate was collected and dissolved in 300 µL of PBS. All samples were stored at -20 ˚C until further analysis.

Using the standard protocol described by Forsgren et al. [7], samples were prepared. Then, DNA extraction was performed using the DNeasy® Mini Kit QIAGEN (Qiagen, Germany). After that, a pair of primers (F: ATCAAGTACAGTTAGTCTTTAG; R: ACGATTCAAAGCTAACTGAATCAGT) was used to amplify the desired fragment of *E. faecalis* [12]. PCR was accomplished using 50 ng of genomic DNA, 10 pmol of each specific forward and reverse primer, and 12.5 µL of Taq DNA Polymerase Master Mix RED 2x (Ampliqon, Denmark) in a final volume of 25 µL.

The PCR comditions for *E. faecalis* were set as follows: initial denaturing at 94 ˚C for 7 min, followed by 34 cycles at 94 ˚C for 40 sec, an annealing step at 46 ˚C for 40 sec, and extension at 72 ˚C for 50 sec. The final extension was set at 72 ˚C for 10 min. Subsequently, the PCR products were analyzed by agarose gel electrophoresis using a 1% agarose gel. Then, the amplified products were visualized with a UV trans-illuminator. For the sensitivity test, specific concentrations of DNA extracted from the standard

E. faecalis were subjected to the corresponding optimal PCR conditions, and the appropriate concentration was considered for the PCR reaction of all collected samples.

2.3. Statistical analysis

To determine the number of apiaries, the sample size was calculated using Cochran's formula [11]. The frequency of infection was calculated for all provinces based on PCR results.

3. Results

Results of bacterial culture for the positive control, the standard bacterium, in a specific medium are shown in Figure 1. Results of the sensitivity test to determine the appropriate concentration for the PCR reaction using serial dilutions (1:100) based on the DNA extracted from the standard *E. faecalis* are shown in Figure 2. Results of PCR amplification for detecting *E. faecalis* in honey samples collected from different provinces of the country are shown in Figure 3.

The results of the PCR method showed that 119(46%) of the 260 samples, were positive for *E. faecalis* (Table 1). In some provinces, all honey samples collected from apiaries were positive for *E. faecalis*, while in some provinces, such as Fars and Golestan, none of the samples were positive for this pathogen in the PCR test.

4. Discussion

Honey bees have positive effects on several different areas, but their key role is in plant pollination [1]. Many factors such as pesticides, harmful environmental conditions, poor nutrition, and diseases have influenced the beekeeping industry and have caused a growing rate of mortality in colonies [13]. *M. plutonius* as a gram-positive bacterium, causes EFB disease in honey bee larvae [14]. Also, there are a few secondary bacteria, for example, *P. alvei*, *E. faecalis*, and *B. laterosporus*, which can attack and harm the bee larvae and may be related to EFB [8]. So, the presence of these bacteria may be considered a remarkable sign for detecting the disease. While the presence of *E. faecalis* like *P. alvei*, has been considered as a possible evidence of EFB disease, the role of such secondary bacterial invaders in disease development has not been well studied [7].

Detection of honey bee diseases is typically accomplished using bee larvae samples. Nevertheless, honey samples can be used as environmental DNA sources [15] from honey bees, pollen, or microorganisms, which allows the detection of infections in honey bees. Using honey samples for the detection of diseases in many areas such as Europe, Asia, Oceania, Africa, and South America has been performed, successfully [15, 16]. In an epidemiological study, about 5.26% of the bee samples

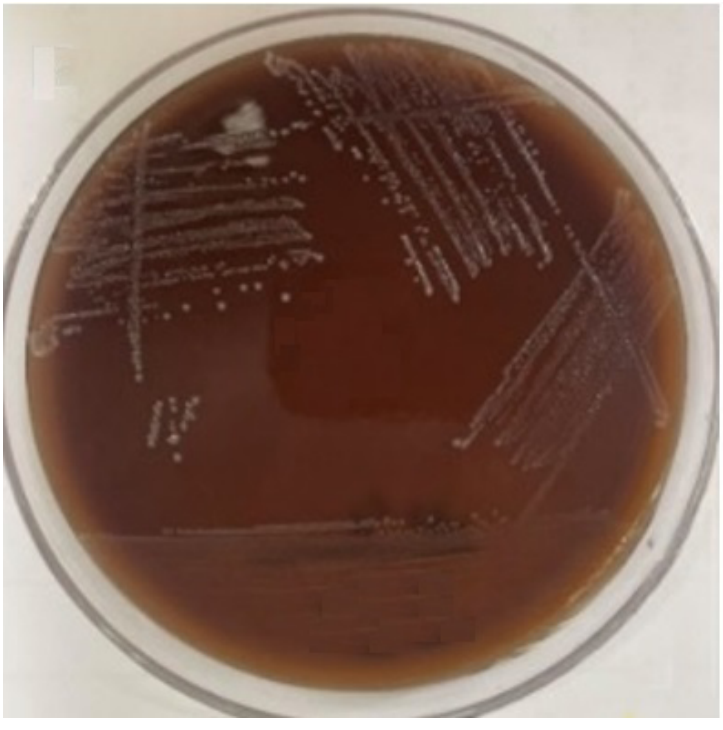

Figure 1. Bacterial culture of the standard *E. faecalis* bacterium in a specific media (blood agar)

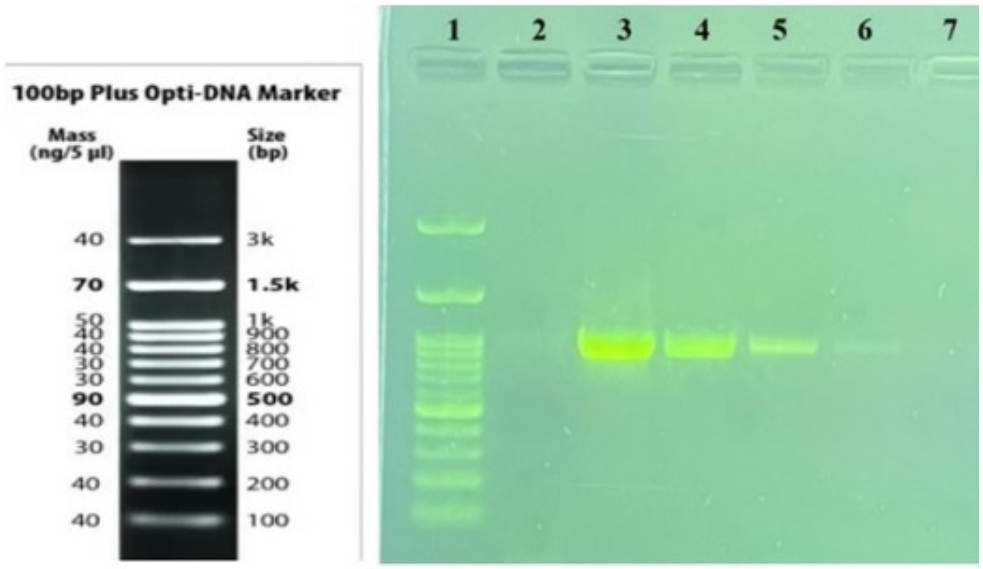

Figure 2. Sensitivity test of DNA extracted from the standard *E. faecalis*

Note: Lane 1: Marker; Lane 2: Negative control; Lane 3: Concentration of 500 ng; Lanes 4, 5, 6 and 7: Concentrations of 5 ng, 500 pg, 5 pg, and 0.05 pg of DNA extracted from the standard *E. faecalis*, respectively.

were positive for *Paenibacillus* larvae, the agent of AFB infection, while 15.78% of honey samples were positive for this pathogen [17]. These results showed that detection of AFB using honey samples may help in improved detection of the disease.

In this study, the distribution of *E. faecalis* was evaluated among colonies using honey samples regardless of the symptoms to find out the distribution of studied bacterium in apiaries of the country. In a study, it has been shown that 31.9% of honey samples were positive for typical *M. plutonius* in a multiplex PCR assay in Japanese honey [18]. We should keep in mind that currently, limited information is available on procedures for diagnosing *M. plutonius* from honey samples. However, McKee et al. [19] used the semi-nested PCR described by Djordjevic et al. [20] and identified *M. plutonius* from honey bee, honey and pollen samples in colonies affected by EFB disease.

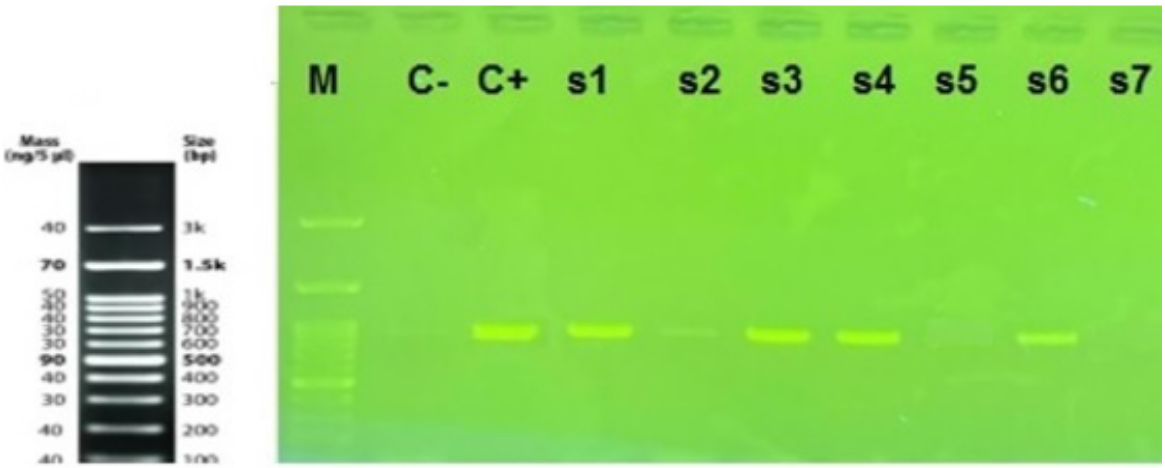

Figure 3. Conventional PCR results for *E. faecalis*

Note: Lane 1: Marker; Lanes 2 and 3 represents the negative and positive controls, respectively, and the remaining lanes (S1-S7) represents the PCR products of positive/negative samples for *E. faecalis* bacterium from different regions.

Table 1. The number of honey samples positive for *E. faecalis* infection among the collected honey samples

Provinces	No. of Apiaries	No. of *E. faecalis* [+]
Alborz	6	6
Ardebil	4	0
Bushehr	3	3
Tehran	10	9
Chaharmahal & Bakhtiari	7	7
East Azarbayijan	15	0
Esfahan	10	0
Fars	21	0
Ghazvin	5	1
Ghom	2	0
Gilan	14	13
Golestan	10	0
Hamedan	10	0
Hormozgan	1	1
Ilam	8	8
Kerman	6	2
Kermanshah	13	11
Khuzestan	5	2
Kohkilouyeh & Buyer Ahmad	8	8
Kordestan	8	1
Lorestan	10	5
Markazi	5	0
Mazandaran	22	4
North Khorasan	7	7
Razavi Khorasan	18	17
Semnan	2	2
Sistan & Baluchestan	6	0
South Khorasan	3	3
West Azarbayijan	17	7
Yazd	2	2
Zanjan	6	0
Total	260	119(46%)

In Italy, Ribani et al. [16] showed that *M. plutonius* was the most common pathogen, with 87% of positive samples using a qualitative PCR method. It is worth mentioning that honey samples can be appropriate for monitoring the presence of pathogens and also for assessing health conditions at the level of the apiary. However, they cannot be useful for the inspection of individual colonies because of the derivation of honey bee from a mixture of several hives [16].

In a previous study, Dehghan et al. [21] showed that *E. faecalis* can be found in bee samples while there was no sign of *M. plutonius*. This may be due to the later growth of *M. plutonius* compared to *E. faecalis*. Accordingly, infection with *E. faecalis* may be another reliable sign for *M. plutonius* diagnosis and EFB disease detection [19]. In a similar sampling method to our study, Dehghan et al. [21] reported lower levels of *E. faecalis* infection in honey bee samples (28.5%). This might indicate the superiority of honey samples in diagnosing *E. faecalis*. Forsgren et al. [7] have stated that honey samples may be a useful tool for identifying sources of *M. plutonius*. They showed that in the absence of *M. plutonius*, *E. faecalis* does not increase in honey bee larvae; thus, the presence of *E. faecalis* in large numbers may be considered possible evidence of EFB disease [22]. In our study, *E. faecalis* was simply detected in honey samples using PCR. This may be because of *E. faecalis* spore formation, which resists the antimicrobial properties of honey.

In apiaries, when we do not detect pathogens, we consider these apiaries to be at a low risk of developing EFB or AFB diseases in that year. So, we can reduce the amount of antibiotics or other treatments [18]. Moreover, it has been shown that environmental factors, like annual temperature and climate, have a relationship with pathogenic infections in honey bees [23]. In a study, Sopko et al. [24] showed a positive correlation between the presence of *P. alvei* and *E. faecalis* along with an increase in *M. plutonius* infection in the worker microbiome [24].

The results of this study showed that the EFB disease in the apiaries of all provinces should be noted as a very major problem, and solutions should be considered to resolve it. It is worth mentioning that due to the overgrowth of secondary bacteria like *E. faecalis*, the presence of this bacterium in large numbers may be considered probable evidence of *M. plutonius* as the main agent of EFB disease. Findings showed that honey samples are more readily available and easier to use in the detection of pathogens, such as *E. faecalis*, which can be simply detected in honey samples.

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Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization and study design: Masoumeh Bagheri and Mojtaba Moharrami; Experiments, data acquisition data interpretation, statistical analysis, and writing the original draft: Masoumeh Bagheri; Review and editing: Masoumeh Bagheri and Naheed Mojgani; Administrative, technical, and material support: All authors.

Conflict of interest

The authors declared no conflict of interest.

Data availability

The data that support the findings of this study are available upon request from the corresponding author.

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