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 which is caused by *Melissococcus plutonius* (*M. plutonius*) and then, the larvae are infected with secondary bacteria such as *Enterococcus faecalis* (*E. faecalis*), *Brevibacillus laterosporus* and *Paenibacillus alvei*. So, the presence of the secondary bacteria can be noted as a remarkable sign for EFB disease. Since preparation of honey bee samples for investigation of apiary health have principal problems, this research aimed to use honey samples to track *E. faecalis* as the secondary agent of EFB disease in apiaries all over Iran. **Material and Method**s: Thus, the number of 260 apiaries were selected and honey samples were collected during Autumn and Winter 2023. After the preparation of the honey samples according to the standard protocol, the genomic DNA was extracted from the samples and then, a pair of specific primer was used to amplify the target fragment of *E. faecalis* using polymerase chain reaction (PCR) technique. For the positive and the negative controls, the standard bacterium and the distilled water were used, respectively. Sensitivity test was performed by specific concentrations of DNA extracted from the standard *E. faecalis* with the corresponding optimal PCR reaction condition and the appropriate concentration was considered for the PCR reaction for collected samples. **Results:** PCR results showed that from 260 honey samples, 119 (46%) samples were positive for *E. faecalis*. **Discussion:** 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 detection of *E. faecalis*. Also, sever infection of the Iranian apiaries with *E. faecalis* as an indicator of EFB disease should be noticed as a very significant problem.

Keywords: *Enterococcus faecalis*, Honey sample, European foulbrood, PCR detection

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 the plants [1] by increase in their diversity, and then ultimately an improve in crop productivity. So, they have a vital key in food security [2]. Though, in recent years a significant increase has been occurred in mortality in managed honey bee colonies around the world [3]. Several agents such as viruses, fungal and bacteria infect honey bees. European foulbrood (EFB) disease is caused by *Melissococcus plutonius* [*M. plutonius*], an anaerobic gram-positive lanceolate bacterium [4, 5]. Brood diseases (EFB and American foulbrood (AFB) diseases) are the most significant reasons for the honey bee larvae death 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 identified on the brood frame. The most observed symptom in larvae, depending on the severance of the disease, is the modification in color from white to yellow, brown, and even greyish-black [7]. Following *M. plutonius*, secondary bacteria; for example, *Enterococcus faecalis* (*E. faecalis*), *Paenibacillus alvei* (*P. alvei*) and *Brevibacillus laterosporus* (*B. laterosporus*) may attack infected colonies [8]. It should be kept in mind that following an *M. plutonius* infection, contamination to the secondary invaders necessarily do not increase disease lethality. It is maybe just a colonization of weak or dead larvae and can be a remarkable sign for EFB disease [9].

Regarding the lack of treatments for the pathogenic based honeybee diseases, the management strategies may be the best way to control these diseases in apiaries. So, detection and elimination of pathogenic agents in hive could help improve its health. Honey sample as a resource of environmental DNA that is easily accessible, can be useful for the detection of the pathogen 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 in all over apiaries in the country.

2. Materials and Methods

2.1. Data collection

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

$$N = z^2 [pq]/d^2$$

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

2.2 Bacteria culture, sample preparation, DNA extraction and PCR

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

Using standard protocol described by Forsgren et al. [7], samples were prepared. Then, DNA extraction was performed by 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 accomplish using 50 ng genomic DNA, 10 pmol of each specific forward and reverse primers, 12.5 μL Taq DNA Polymerase Master Mix RED 2x (Ampliqon, Denmark) in a final volume of 25 μL.

The condition of PCR for *E. faecalis* was set as an initial denaturing at 94 °C for 7 min, followed by 34 cycles at 94 °C for 40 sec, annealing temperature at 46 °C for 40 sec and extension for 50 sec at 72 °C. Final extension was set at 72 °C for 10 min. Subsequently, by agarose gel electrophoresis using 1% agarose gel, the PCR products were observed. Then, the amplified products were visualized with UV trans-illuminator. For sensitivity test, specific concentrations of DNA extracted from the standard *E. faecalis* were subjected to the corresponding optimal PCR condition, and the appropriate concentration was considered for the PCR reaction for all collected samples.

2.3. Statistical analysis

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

3. Results

Results of bacterial culture for positive control, standard bacterium, in specific media are shown in Figure 1. Results of sensitivity test to determine the appropriate concentration for 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 some honey samples from different provinces of the country are shown in Figure 3.

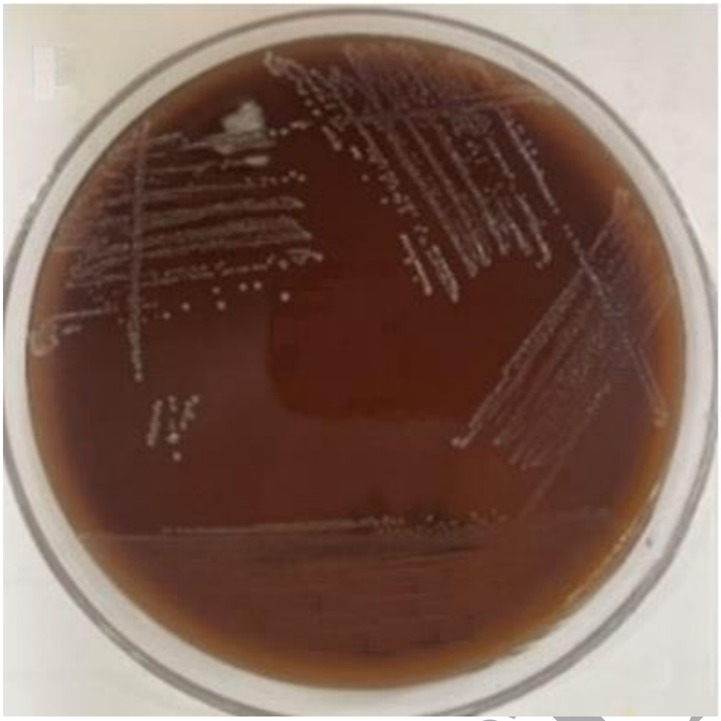

Figure 1. Bacterial culture for standard *E. faecalis* bacterium in specific media (blood agar).

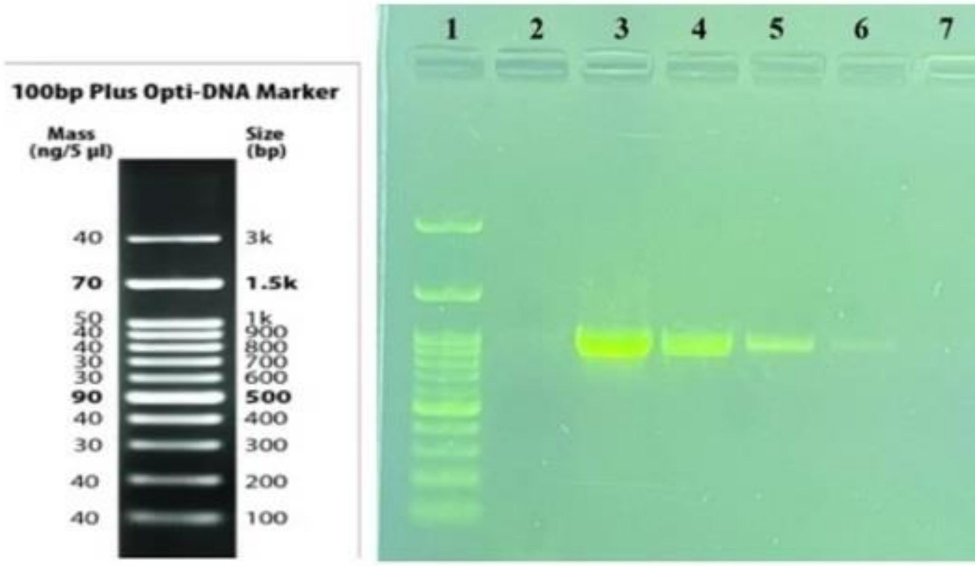

Figure 2. Sensitivity test of DNA extracted from the standard *E. faecalis*; Lane 1: marker, lane 2: negative control, lane 3: concentration of 500 ng; Lanes 4, 5, 6 and 7: concentration of 5 ng, 500 pg, 5 pg and 0.05 pg of DNA extracted from the standard *E. faecalis*, respectively.

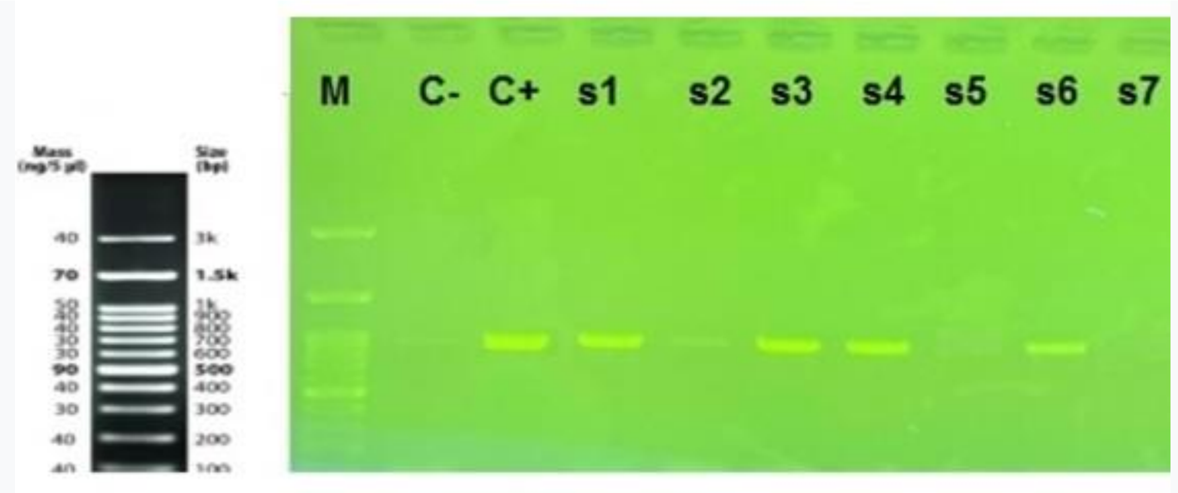

Figure 3. Conventional PCR results for *E. faecalis*; Lane 1: marker, lanes 2 and 3 are the negative and positive controls, respectively, and other lanes including s1 to s7 are the PCR products of positive/negative *E. faecalis* bacterium in different regions.

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

Table 1 The number of positive honey samples for *E. faecalis* infection in the 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%)

4. Discussion

Honey bees have positive effects on several different areas, but their key role is in plants pollination [1]. Many factors such as pesticides, harmful environmental conditions, poor nutrition and diseases have influenced the beekeeping industry and have caused growing rate of death 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 with EFB [8]. So, the presence of these bacteria may be noted as 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 lets 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 have been performed, successfully [15, 16]. In an epidemiological study, about 5.26% of the bee samples were positive for *Paenibacillus larvae*, the agent of AFB infection, while 15.78% of honey samples were positive for this pathogen [17]. This results showed that detection of AFB using honey samples may help in enhanced detection of the disease.

In this study, the distribution of *E. faecalis* were evaluated among colonies using honey samples regardless of the symptoms to find out the release 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 multiplex PCR assay in Japanese honey [18]. We should keep in mind that currently; limited information is presented 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.

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

In previous study, Dehghan et al. [21] shown that *E. faecalis* can be found in bee samples while there was no sign of *M. plutonius*. This may be due to late growth of *M. plutonius* than *E. faecalis*. Accordingly, infecting to *E. faecalis* may be another reliable sign for *M. plutonius* diagnosis and EFB disease detection [19]. In similar a 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, so, the presence of *E. faecalis* in large numbers may be considered as possible evidence of EFB disease [22]. In our study, *E. faecalis* showed can be simply detected in honey samples using PCR. This may be because of *E. faecalis* spore formation which resists the antimicrobial properties of honey.

In the apiaries, when we don't detect pathogens, we can be considering these apiaries at a low risk of developing EFB or AFB diseases in the 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 increase in *M. plutonius* infection in the worker's 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 as the probable evidence of *M. plutonius* as the main agent for EFB disease. Findings showed honey samples are more readily available and easier to use in the detection of pathogens like *E. faecalis* which can be simply detected in honey samples.

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Authors' contributions

Study concept and design: M.B and M.M

Acquisition of data: M.B

Analysis and interpretation of data: M.B

Drafting of the manuscript: M.B.

Critical revision of the manuscript for important intellectual content: M.B and N.M.

Statistical analysis: M.B.

Administrative, technical, and material support: M.B, M.M and N.M.

Ethics

Not applicable.

Conflict of interest

All authors declare that there is no conflict of interest.

Data Availability

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

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