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Epidemiological study for detection of the main and secondary agents of European foulbrood disease in the apiaries of Iran

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Abstract

Infecting to *Melissococcus plutonius*, the primary cause of the European foulbrood (EFB) disease, can be followed by infecting to the secondary bacteria, such as *Enterococcus faecalis* and *Brevibacillus laterosporus*. The aim of this research was to diagnose EFB disease by tracking the causes of the disease in apiaries all over Iran. From 260 apiaries, honey bee samples were randomly collected. After samples preparation, the genomic DNA was extracted and specific primers were selected for interested bacteria. Using the conventional polymerase chain reaction (PCR) method for *E. faecalis* and *B. laterosporus* and nested-PCR method for *M. plutonius*, the target fragments were amplified. Desired standard bacteria and distilled water were used as positive and negative controls, respectively. Results showed that out of 260 samples from apiaries, 74 and three samples were positive for *E. faecalis* and *B. laterosporus* bacteria, respectively. Also, the results of nested-PCR showed that 58 samples were positive, of which only 12 samples were positive in the evaluation of *E. faecalis*. Results demonstrated that the highest and the least levels of the infection for *M. plutonius* and *E. faecalis* were in the south and east of the country, respectively. Results indicated that sometimes due to the excessive growth of secondary bacteria, the main bacteria can be removed from the environment. Also, findings proposed that those provinces with higher number of populations, followed by higher amount of air pollution, had more infected samples than others.

Introduction

Honeybees as one of the most important insects in nature, play a key role in supplying many precious pollination services for a vast variety of agricultural crops, being a crucial stage in almost 300,000 flowering plants species' reproductive cycle.[1] There are many different agents against honeybees, like more than 30 viruses, most widespread in apiculture,[2] and fungal and bacterial diseases, such as chalkbrood (caused by *Ascosphaera apis*), stonebrood (caused by *Aspergillus spp*.), and American foulbrood and European foulbrood (EFB), being caused by Gram-positive bacteria, *Paenibacillus larvae* and *Melissococcus plutonius*, respectively.[3-6] Indeed, brood diseases are one of the most important reasons for the death of honeybee larvae, leading to the weakening and eventually death of infected colonies.[4] Moreover, microsporidia, like *Nosema ceranae*, can also be a threat to honeybees,[7,8] and parasites are the most harmful pathogens for them that can cause colony loss. The *M. plutonius*, an anaerobic Gram-positive bacterium, causes EFB in honeybees. Although EFB occurs worldwide, it is not easily notifiable in all countries yet. A colony attacked by EFB shows capped and uncapped cells, being detected irregularly sporadic over the brood frame. Speaking of symptoms, the color of larvae suffering from EFB alters from white to yellow, brown, and even greyish black, depending on the disease severity, respectively.[9] The larval gut is where *M. plutonius* reproduces in, so survived larvae can infect other larvae by their feces when they pupate.

Based on the recent report of Ministry of Agriculture, there are more than 94,000 beekeepers and more than 10 million honeybee hives, and more than 136 kton of honey is produced annually in Iran. However, beekeeping has been impressed by various factors, like pesticides, damaging environmental conditions, and poor nutrition, all of which in combination with diseases exert a synergistic negative effect, growing the number of colonies' death.[10]

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There are several secondary bacteria, such as *Enterococcus faecalis*, *Paenibacillus alvei*, and *Brevibacillus laterosporus*, which can attack infected colonies following a *M. plutonius*.[11] Although infecting to the secondary attackers following a *M. plutonius* infection does not boost disease lethality, it is a remarkable sign for EFB detection.[12]

Considering the reaction sensitivity increase in nested-polymerase chain reaction (PCR)[13] using two serial rounds of PCR, this method is proposed for diagnosing *M. plutonius*.[14] In this method, each round of PCR has its own set of primers and the product of the first reaction is used as a template for the second one. In this research, the presence of the primary and secondary agents for EFB was evaluated using hemi-nested and conventional PCR in adult bees in all apiaries of Iran.

Materials and Methods

Data collection and sample preparation. Sampling was performed randomly based on the table of the number of apiaries in the country by province and prevalence rate of 40.00%, being reported by Iran Veterinary Organization. Population size was evaluated using the Cochran's formula:[15]

$$N = z^2(pq)/d^2$$

where, *d* or error was equal to 0.06, *p* was equal to 0.40, *q* was equal to 0.60, and *z* or the normal variable was equal to 1.97 with a confidence factor of 95.00%. Therefore, N ≅ 260. 260 apiaries from 31 provinces of the country were considered, whereas for each apiary, a number of hives were randomly selected, and regardless of the clinical symptoms of the disease, a number of bees were collected from each comb in the sterile containers (more than 50 adult bees for each apiary). After that, all collected adult bee samples from each apiary were pooled and sent to the laboratory using a cold chain to check the bacteria. Beekeepers were asked about their colony losses.

Bacterial culture. According to the Forsgren *et al*.,[9] for positive control, standard bacteria (provided by Razi Vaccine and Serum Research Institute, Karaj, Iran) were cultured in specific media including Baily agar and blood agar (Biolab Inc., Budapest, Hungary) for *M. plutonius* and secondary bacteria, respectively.

DNA extraction and PCR. After preparing samples as described by Forsgren *et al*.,[9] DNA extraction was performed using DNeasy® Mini Kit (Qiagen, Hilden, Germany). After that, two pairs of primers for secondary bacteria and three primers for *M. plutonius* were performed to amplify desired fragment of interested bacteria (Table 1). The PCR was accomplished using 50.00 ng genomic DNA, 20.00 pmol of each specific forward and reverse primers, and 12.50 µL Taq DNA Polymerase Master Mix RED 2X (Ampliqon, Odense, Denmark) in a final volume of 25.00 µL. For *M. plutoinus*, the condition of hemi-nested-PCR for external fragment was set as an initial denaturing at 95.00 ˚C for 2 min, followed by 40 cycles of 95.00 ˚C for 30 sec, annealing temperatures of 61.00 ˚C ˚C for 15 sec, and extension at 72.00 ˚C for 1 min. The final extension was set at 72.00 ˚C for 5 min. After confirming the correctness of the external fragment, PCR was carried out for the internal fragment using the PCR product of the first round. The PCR was set as an initial denaturing at 95.00 ˚C for 2 min, followed by 25 cycles of 95.00 ˚C for 30 sec, annealing temperatures of 56.00 ˚C for 15 sec, and extension at 72.00 ˚C for 1 min. The final extension was set at 72.00 ˚C for 5 min. Finally, PCR products were monitored by 1.00% agarose gel electrophoresis. The condition of PCR for both secondary bacteria is shown in Table 2. The PCR products were monitored by 1.00% agarose gel electrophoresis. It should be noted that specificity and sensitivity of primers for all bacteria were analyzed. After that, all 260 samples were tested for *M. plutonius* using hemi-nested-PCR and for secondary bacteria, using conventional PCR. The PCR products were observed by agarose gel electrophoresis using 1.00% agarose gel. Ultra-violet light of a trans-illuminator was used to visualize the amplified product. Positive samples were cultured in specific media to isolate the bacteria.

Table 1. Primer sequences used for simple and nested-polymerase chain reaction.

Bacteria	Sequences	Size	Target	References
Melissococcus plutonius	MP1: F: CTTTGAACGCCTTAGAGA MP2: External R: ATCATCTGTCCCACCTTA MP3: Internal R: TTAACCTCGCGGTCTTGCGTCTCTC	486 276	16S rRNA	15
Enterococcus faecalis	F: ATCAAGTACAGTTAGTCTTTAG R: ACGATTCAAAGCTAACTGAATCAGT	949	16S rRNA	16
Brevibacillus laterosporus	F: AGTCAAAATGATCTTATTGTCAGCATCG R: CATCTTTTCAATGAAATTCCCTTGAG	1,200	ftsA	17

Table 2. The condition of polymerase chain reaction for *Enterococcus faecalis* and *Brevibacillus laterosporus.*

Bacteria	Initial denaturation	Cycle	Denaturation	Annealing	Extension	Final extension
Enterococcus faecalis	94.00 ˚C, 7 min	34	94.00 ˚C, 40 sec	46.00 ˚C, 40 sec	72.00 ˚C, 50 sec	72.00 ˚C, 10 min
Brevibacillus laterosporus	94.00 ˚C, 3 min	35	94.00 ˚C, 30 sec	55.00 ˚C, 45 sec	72.00 ˚C, 45 sec	72.00 ˚C, 90 sec

Statistical analyses. To determine the number of apiaries for sampling, population size was evaluated using Cochran's formula.[15] Results of molecular process were evaluated by descriptive statistics. Also, the chart of bacteria distribution was made by Adobe Illustrator Software (version 10.0; Adobe, Mountain View, USA).

Results

Results of bacterial culture for positive controls and standard bacteria in specific media (Baily agar and blood agar for *M. plutonius* and secondary bacteria, respectively) are shown in Figure 1.

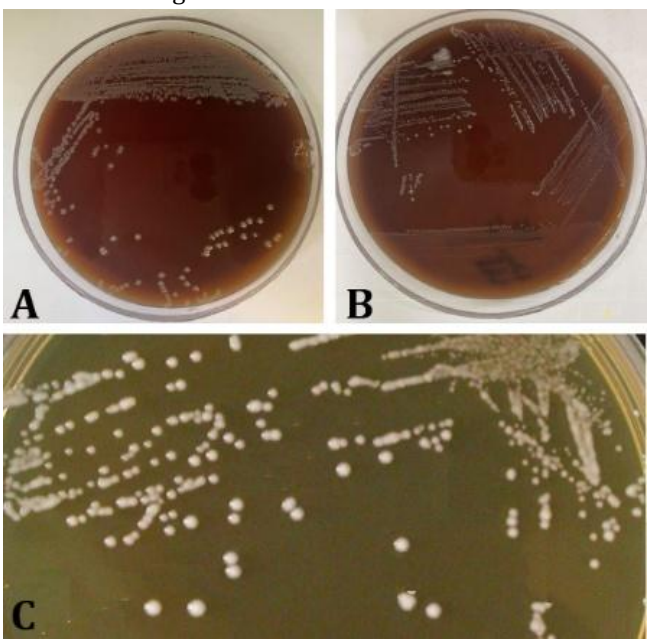

Fig. 1. Results of bacterial culture for studied standard bacteria in specific media. **A:** *Brevibacillus laterosporus*; **B:** *Enterococcus faecalis*; **C:** *Melissococcus plutonius*.

The results of molecular detection showed that out of 260 samples, for *M. plutonius*, 58 (22.30%) samples and for *E. faecalis*, 74 samples (28.46%) were positive. Twelve samples were found positive for *M. plutonius* and *E. faecalis*, simultaneously. Three of all samples were positive for *B. laterosporus*, which none of them was positive for *M. plutonius*. Among all 58 positive samples for *M. plutonius*, the frequency of provinces with only one infected sample was the highest. Considering the fact that the presence of secondary bacteria indicates EFB infection, 135 samples were classified as positive, which 12 of them were found to be common. So, 123 (47.30%) samples from 31 provinces were infected by EFB. Additionally, all provinces except one were infected with at least one of the bacteria. It was found that among all the samples collected from all over the country, 22.30% of the samples were infected with *M. plutonius*, 28.50% with *E. faecalis*, and 1.15% with *B. laterosporus*.

Figure 2 shows the distribution of primary and secondary bacteria all over the country. Although the number of hives differs in each province, this map helps us evaluate the rate of infection considering the area of each province. Totally, north-west of the country had more infected samples than the other parts. Assuming that the provinces of the country were divided into five groups, including north, south, east, west, and center, 25.86% of bees infected with *M. plutonius* were from the northern provinces of the country. While the highest level of infection was in the south of the country, the least level of infection was belonged to the east of the country. Similarly, the highest infection with *E. faecalis* was in the northern provinces of the country, and the least of that was belonged to the east of the country. Figure 3 shows the results of comparing different parts of the country for *E. faecalis* and *M. plutonius* infections.

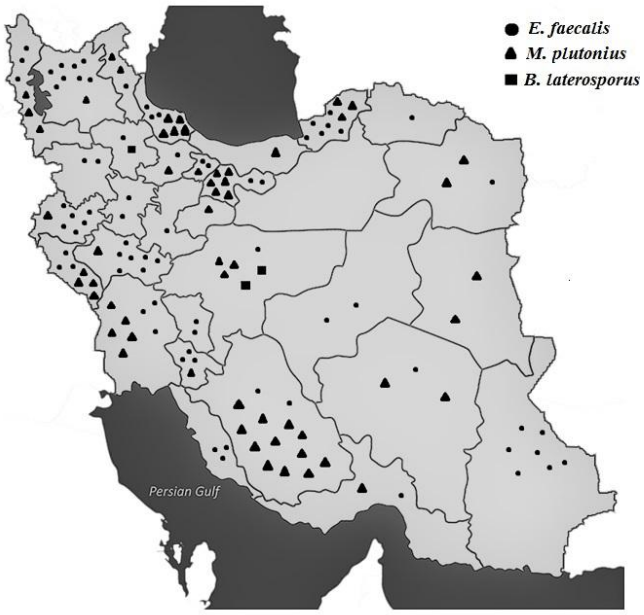

Fig. 2. The distribution of primary and secondary bacteria all over the country.

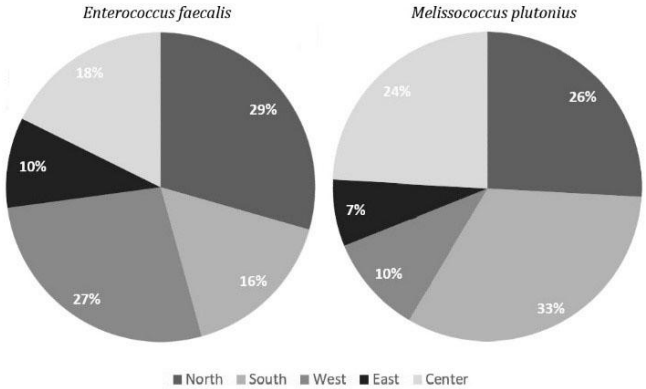

Fig. 3. Results of comparing different parts of the country for *Enterococcus faecalis* and *Melissococcus plutonius* infections.

Discussion

Honeybees are well-known for their positive effects on numerous different areas. They not only produce honey, beeswax, royal jelly, and propolis but they also play a key role in plants pollination,[18] particularly commercial pollinations of some crops. However, over the recent years there have been dramatic surges in losses in managed honeybee colonies in some parts of the world.[19] There are several biotic and abiotic factors adversely impacting bees' welfare and survival, such as changes in land use and management intensity, climate change, beekeeper's management practices, lack of forage (nectar and pollen), use of pesticides in agriculture, and parasites and pathogen infections.

Severe colony losses have been occurring since the year of 2006 in the world. A Gram-positive bacterium, *M. plutonius*, causes EFB disease in honeybee larvae,[20] and is prevalent in most beekeeper countries.[21] The disease is scattered widespread, causing brood losses and consequently colony collapse.[22] The digestion of contaminated food derives the proliferation of the pathogen in the larval mid-gut, leading to the consumption of the larval food. Thus, diseased larvae die from starvation not from the infection itself.[20] Symptoms of EFB are recognized by visual examination of brood combs, consisting 4-5-day-old dead larvae.[22] The impact of secondary agents is one of the factors influencing the velocity of pathogens.[23] For EFB disease, there are a few secondary bacteria attacking the larvae, such as *P. alvei*, *E. faecalis*, and *B. laterosporus*, all of which might increase the possibility of harming the larvae.[11] Therefore, secondary bacteria are another remarkable sign for the disease detection.

Several various methods have been used for detecting *M. plutonius*, such as bacterial staining,[24] enzyme-linked immunosorbent assay,[25] PCR[15], and culture of the bacterium from diseased larvae.[22] Forsgren *et al*.,[26] have illustrated a non-inclusive presence of *M. plutonius* in larvae and honey in the brood nest emanating from colonies with and without symptoms of EFB. In this research, the distribution of *M. plutonius* among colonies was evaluated asymptomatically, utilizing samples of adult bees to figure out the dispensation of the bacteria in the host population. The highly sensitive hemi-nested-PCR for *M. plutonius* and conventional PCR for secondary bacteria were also used; both methods were established by Forsgren *et al*.[9]

Although EFB occurs worldwide, it is not easily detectable in all countries. Accordingly, honeybee has been studied several times in Australia, and the occurrence has not yet been reported in New Zealand.[4,24] However, it is more common in Europe, being the most common bacterial brood disease in United Kingdom.[27] In Switzerland, EFB incidence has increased steadily every year since the late 1990s, even if diseased colonies have been eradicated.[28] According to Mayack and Hakanoğlu,[29] foulbrood diseases occurred frequently in Türkiye, with the infection rate exceeding 60.00% in some regions in autumn. Investigation of EFB in Canadian commercial beekeeping operations revealed that there are two sequence types of *M. plutonius* from EFB outbreaks.[30]

It has been reported that various factors are involved in the virulence of *M. plutonius*, such as honeybee strains, rearing conditions of the larvae, observation period, inoculum doses, and even differences in the strains of *M. plutonius*.[31] Until this research, *M. plutonius* had not been detected using molecular methods and secondary bacteria in Iran. Forsgren has explained that the detection of infectious microorganisms by PCR has been revolutionized and described as a gold standard for the detection of some microbes.[11] The use of on-site visual diagnostics alone is not considered sufficiently reliable; therefore, it is legally required to confirm EFB using an appropriate diagnostic method. Findings of Forsgren *et al*.,[26] showed that *M. plutonius* can be found in larvae in all bee colonies with EFB symptoms, but in all colonies classified as EFB cases in the field, healthy-appearing larvae from such colonies were examined. However, it was not detected at all. The presence of bacteria in the offspring was not limited to the symptomatic larvae, but they were found mostly in the diseased larvae. In many cases, apparently healthy larvae failed to amplify the product by PCR. The *M. plutonius* could be detected only in 35.00% of hives from clinically diseased colonies.[26] Even if secondary bacteria can be found in samples not showing signs of *M. plutonius*, this does not mean that the samples are not infected with *M. plutonius*. Indeed, it indicates that the secondary bacteria have been displaced due to the lack of food and slow growth of *M. plutonius*. Therefore, being infected with secondary bacteria is another reliable sign to identify the *M. plutonius* and diagnose EFB, being also used in this study to find the disease accurately and correctly.

Based on redundancy analyses of the microbiome composition, it has shown that the detection limit-based qPCR of *M. plutonius* uncovered a more noteworthy changeability within the microbiome profiles of worker bees compared with those explained by clinical signs and PCR detection. It became clear that the fluctuations were larger. The presence of secondary invaders (*P. alvei* and *E. faecalis*) was positively correlated with an increased profile of *M. plutonius* in the worker microbiome.[32] Asymptomatic colonies from diseased apiaries were shown to have a higher risk of being positive for *M. plutonius* compared to the asymptomatic colonies from disease-free apiaries. If large amounts of *M. plutonius* are detected in adult bees and larvae, the sample is likely to have symptoms.[33] Microbial profiles obtained by denaturing gradient gel electrophoresis have shown no relevant differences between samples, suggesting that

healthy-appearing samples are partially affected. The study showed that honeybee larvae were affected by EFB and an atypical *Paenibacillus* species emerged as a second invader, possibly imparting different symptoms to the affected larvae.[34]

In this research, in terms of the priority and importance of handling the provinces of the country, the infection levels of the provinces in relation to the number of samples were compared. Since the provinces of Khuzestan, Yazd, Hormozgan, Sistan and Baluchistan, and Bushehr were 100% positive for one of the bacteria (*M. plutonius* or *E. faecalis*) in all samples; so, they should be in the priority of further investigations. In the next places, the provinces of East Azerbaijan, South Khorasan, Golestan, Lorestan, and Fars with more than 50.00% of positive samples are very important in terms of the need for treatment.

There are many hypotheses about the different numbers of infected samples in various provinces, like being in a specific weather condition, air pollution, windy weather, hot or cold regions, humidity, management intensity, and diseases. The fact is that, all of the hypotheses mentioned above are possible and even could affect colonies altogether. The incidence of EFB was found positively correlated with temperature (r = 0.341), and negatively correlated with rainfall (r = – 0.144) and relative humidity (r = – 0.494), though non-significant.[35] Thus, humid provinces showed almost a smaller number of infected samples. Further, samples from south of Iran had higher percentages of infection than other regions; although, the provinces had a smaller number of samples. Indeed, hot climate regions showed more vulnerability to EFB than cold climate ones.[35] Roy and Franco[36] also released their results validated the Jangir and Yadav findings.[35] The original infection spreads through the air because the tiny spores of rotten brood ejected from the hives are dispersed by the wind, carried to the honey or pollen, and infect the larvae through their food. Some regions were more susceptible for spreading EFB because of the wind, elucidating the importance of the environmental conditions during the bee management. This study was limited to diagnose infected samples, but could be extended for the multi-factorial reasons of EFB, mentioning other environmental conditions, such as air pollution. As it was observed in the present study, those provinces with the higher number of populations, followed by the higher amount of air pollution, had more infected samples than others. Since several issues remained unaddressed, a future extension is suggested to find out why honeybees are dealing with EFB in various conditions. One hypothesis could bethat the colonies are susceptible to get sick because of becoming weak after being involved in another disease. Overall, all of the hypotheses need to be tested to be valid. However, the fact is that, they could all be a cause of the disease.

In order to improve the diagnosis process of this disease, it is suggested to include the identification of *M. plutonius* strains and determine their virulence in the future proposed plans, because it can be of great help in the diagnosis and identification of the disease both inside and outside of the country. Investigating atmospheric and geographical conditions is one of the other ways which their effects have not been investigated in detail and can be one of the important candidates for increasing/decreasing the amount of infection. Also, in order to reduce the overall rate of disease in the country and apiaries, it is better for the beekeepers to take preventive measures by removing the weak populations and disinfecting the hives and related necessities. Indeed, disease prevention can be performed in different ways; the best way is to keep the bees' habitat clean. Identifying the centers of disease can also be very helpful in reducing the spread of disease and subsequently controlling the long-term use of antibiotics in sick colonies.

In general, it is suggested that beekeepers prevent the spread and deterioration of the disease by taking preventive measures, because if even a small population of bees gets sick, the disease can quickly spread to the colony level and reach the point of death of the entire colony. Among these measures, the creation of a colony in areas rich in nectar and pollen, changing old and contaminated frames, changing the queen every 2 years, refraining from buying hives and necessities from unknown sources, monitoring epidemiological investigation of EFB disease using the PCR diagnostic method, and identifying the contamination of bee export products, especially honey, by PCR method and also issuing health certificates for them can be mentioned.

In conclusion, *M. plutonius*, the primary bacterium, could be found in symptomatic and asymptomatic samples sharing the same habitat with secondary bacteria. In some cases, *M. plutonius* is not detectable; thus, secondary bacteria could be a good marker for EFB as well. The total number of infected samples differs according to the duration of infection, colony managements, hygiene, and environmental conditions.

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Conflict of interest

All authors declare that there were no interests or conflicts of attribution.

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