Harran Üniv Vet Fak Derg, 2021; 10 (1): 56-59 *Research Article*
DOI:10.31196/huvfd.881082

Investigation the Exsistence of European Foulbrood Disease in Larvae and Adult Honeybees in Some Regions of Turkey with Conventional PCR Methot*

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Geliş Tarihi: 15.02.2021 **Kabul Tarihi: 12.04.2021**

Abstract: European foulbrood disease caused by *Melissoccocus plutonius* which gram-positive bacterium that leads to larval deaths with its worldwide prevalence, paving the way for beehives to die away over time. It causes economic losses in honey production. Bee breeding and honey production are economically pivotal in Turkey, and it is vital to analyze the prevalence of this disease. This study aims to identify *M. plutonius* from the honeycombs suspected of European foulbrood and dead bee samples from the beehives (11 hives in total) that contain honeycombs collected from Cankırı, Hatay, Kars and Konya via conventional PCR method. In this study two samples DNA identified as *M. plutonius* (18% of total hives).
Key Words: European foulbrood, Honey bees, *M. plutonius*, Turkey.

Türkiye'nin Bazı Bölgelerindeki Larva ve Yetişkin Bal Arılarında Avrupa Yavru Çürüklüğü Hastalığı Varlığının Konvansiyonel PCR Yöntemi ile Araştırılması

Özet: Avrupa yavru çürüklüğü hastalığı *Melissoccocus plutonius* Gram-pozitif bakteri etkenli bir hastalık olup dünya çapında yaygınlığıyla larva ölümlerine yol açmakta, arı kovanlarının zamanla sönmesine ve bal üretiminde ekonomik kayıplara yol açmaktadır. Özellikle arı yetiştiriciliği ve bal üretiminin ekonomik olarak önem arz ettiği ülkemizde bu hastalığın yaygınlığının araştırılması önemini korumaktadır. Bu araştırmada Çankırı, Hatay, Kars ve Konya illerinden toplanan Avrupa yavru çürüklüğü şüpheli petek ve bu petekleri içeren kovanların (toplam 11 kovan) önünden alınan ölü arı örneklerinden konvansiyonel PCR yöntemiyle bu hastalık etkeni *M. plutonius*'un varlığı tespit edilmeye çalışıldı. Alınan kovanlara ait örneklerin 2'sinde (toplam kovanların %18'inde) *M. plutonius* varlığı tespit edildi.
Anahtar Kelimeler: Avrupa yavru çürüklüğü, Bal arıları, *M. plutonius*, Türkiye.

Introduction

European foulbrood disease is a larval disease caused by *M. plutonius* gram-positive bacterium and observed in bee colonies (Belloy et al., 2007). *Melissoccus plutonius* is a lancet-shaped bacterium that can be seen via an anaerobic microscopic view. In plenty of issues are indicated that *M. plutonius* spreads to every continent where honey bees live, and this disease agent is transmitted to the larvae by adult bees during feeding (Budge et al., 2010).

European foulbrood infects the larvae in the colony, particularly in countries where honey production is common, paving the way for the colony to collapse, thereby leading to severe economic losses. In an epidemiological study conducted by Forgsen et al. (2005) in Sweden, they observed that the disease incidence increased for no reason in 2005. There is insufficient information about the diseases' epidemiology.

Honey bee diseases are categorized as viral, parasitic, bacterial, and fungal, and such diseases often lead to severe consequences. Researches reveals that one of the most vital diseases inducing larvae is European foulbrood disease (Waite et al., 2003). The common application of antibiotics in bee colonies by producers to eliminate bacterial and parasitic diseases threatens people's health that consumes honey obtained from such bee colonies. It leads to the formation of bacterial strains resistant to antibiotics and the spread of this disease (Barganska et al., 2011).

China ranks the top with 543.000 tons of honey production, and Turkey takes second place with 115.000 tons annually per a report published by the Institute of Agricultural Economics and Policy Development in 2020 (Agricultural Economy and Policy Development Institute, 2020). Considering such figures, identifying the prevalence of bee diseases and combating such diseases is vital for both the manufacturer and the country's economy.

While *M. plutonius*'s epizootiology is not entirely understood, which infects honey bee colonies and causing European foulbrood, It is observed that the larvae infected with this bacterium die rapidly are two and three days old.

Besides, *M. plutonius*, causing European foulbrood disease, *Achromobacter Eurydice*, *Brevibacillus laterosporus, Enterococcus faecalis*, and *Paenibacillus alvei* are identified in the colonies during the development of this disease. However, it is unclear what role they play in developing this disease (Alippi, 1991). It aims to identify *M. plutonius* via the conventional PCR method, which causes European foulbrood in honeycombs and bees obtained in some Turkey provinces.

Material and Methods

Local ethics comitte permission: The materials used in this study are not subject to the permission of HADYEK according to the article 8. of the regulation published in the Official Gazette dated February 15, 2014.

Cultural and molecular techniques are frequently employed in identifying the *Melissococcus plutonius*. In many study were indicated that each method has advantages and disadvantages. *M. plutonius* diagnosis is usually based on the combs' color and smell; however, such symptoms can be easily confused with other abnormalities and diseases. It were noted that the cultural methods used for identifying *M. plutonius* have some difficulties as they allow secondary bacteria to grow in the environment (Arai et al., 2012).

In May and June collected dead bee samples of larvae and hives from beehives of beekeepers in Hatay, Konya, Çankırı and Kars provinces (**Kars**: 150-beehive business: 1 suspect, 200-beehives business: 1 suspect, 500-beehives business: 1 suspect; **Cankiri**: 20-beehives business: 1 suspect, 10-beehives business: 1 suspect, 20-beehives business: 1 suspect, 35-beehives business: 1 suspect; **Hatay**: 250-beehives business: 2 suspects; **Konya:** 1 suspect from each of the 50 and 45 beehives business: total 11 suspect hives*)*. We obtained one piece from each of the combs containing *M. plutonius* suspicious dead larvae (yellow, brown, and foul odor), placed them in locked bags, and stored them at -20 ° C examination in the laboratory. We placed adult dead bees in front of the same hive in different locked bags and stored them under the same conditions.

Figure 1. Suspected larvae and honeycomb samples.

Molecular Identification: Adult bee and larvae samples were taken into disposable falcon tubes containing 1 ml of physiological saline and homogenized (adult bees were dissected) (Budge et al., 2010). Qiagen DNA for the DNA extraction kit was employed, and the extraction was conducted per the manufacturer's recommendation.

Concentration for PCR reaction: 2.5 µl $MgCl_2$, primers 1µl (from each primer),

Primer 1: 5' GAAGAGGAGTTAAAAGGCGC 3',

primer 2: 5' TTATCTCTAAGGCGTTCAAAGG 3',

0.5 µl deoxynucleoside triphosphate (dNTP), buffer 2.5 µl, DNA 2.5 µl, RNase and DNase free water 14.5 µl, Taq DNA polymerase was optimized to 0.5 µl.

PCR conditions: starting; 95°C (1min), chain elongation; 93°C (1 min), 55°C (30 sec) and 72°C (1 min), 30 cycles, the last cycle was applied at 72°C (5 min) using a Bio-Rad gradient heat machine. The positive control sample was obtained from the Kafkas University Microbiology Laboratory, and DNase-RNase-free water was used for negative control. The horizontal gel electrophoresis technique was used to visualize the PCR products. The PCR products were placed in 0.8% agarose gel wells stained with ethidium bromide and run in an electrophoresis tank containing TBE buffer solution at 110 volts and 300 milli-ampere for 40 minutes. The bands formed were compared with the hyper

ladder 1 kb, and the resulting DNA fragments were imaged on the UV transilluminator. The resulting images were photographed and documented. Bands corresponding to 812 base pairs (bp) rated as *M. plutonius* positive (Govan et al., 1998; Tibata et al., 2018).

Results

The analysis of the electrophoresis images reveals that *M. plutonius* positive bands were observed from 1 suspicious larva sample taken from the honeycombs in Hatay and one dead bee sample taken from 11 combs in Çankırı in total from Çankırı, Konya, Hatay and Kars (in 18.18% of combs, suspicious of *M. plutonius*) (Figure 2. and Table 1).

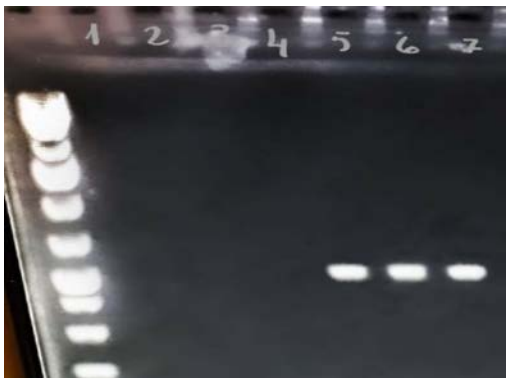

Figure 2. Electrophoresis image (4. Negative control, 5. Positive control, 6. and 7. (812 bp.) *M. plutonius* positive)

Table 1. sampling and PCR findings

Regiones where samples were collected	Number of hives in enterprices	Number of suspected samples	Findings of PCR
Çankırı	20	1	1
	10	1	
	20	1	
Hatay	35	1	1
	250	2	
	150	1	
Kars	200	1	0
	500	1	
Konya	50	1	0
	45	1	
Total	1,255	11 (0.876 %)	2 (18%)

Discussion and Conclusion

It is known that honey bee breeding, which is one of the most widespread insect breeders globally, is vital in ecological aspects besides its economic dimension, considering the economic value of bee products and the role of bees in the pollination of plants. *M. plutonius*, a European foulbrood disease agent, infects the larvae during colonized feeding in the intestinal of adult bees and paving the way for dying out of young colonies. Today, antibiotics are used in treating this disease. However, the use of such antibiotics is prohibited by some countries as it is not a definitive solution in treating this disease, leaves residue in the honey, and causes more resistant strains (Grossar et al., 2020).

It is observed that *M. plutonius* causes European foulbrood, infects honeybee larvae, and dies in combs. It paves the way for morphologically normal-looking larvae to bent at the bottom of the combs, their color shift from pearl white to yellow, then brown and grey-black (Roetschi et al., 2008).

Identifying the presence of *M. plutonius* in brown and malodorous honeycombs in this study supports the literature studies.

Determining the presence of *M. plutonius* in the laboratory is extremely important for confirming European foulbrood disease in honey, larvae and adult bees. The diagnosis of European pup rot disease can be determined by breeders from the change in color and smell that occurs in dead larvae but it is noted that these symptoms are quite insufficient to make a definitive diagnosis. In a study conducted by Rana et al. in India, investigated with PCR methot, they noted that only 15% were positive of bee colonies infected with *M. plutonius* had a characteristic yellow color and vinegar odor (Rana et al., 2012).

Finding 18% of the suspicious hives with *M. plutonius* as positive is similar to the results found by Rana et al. 2012.

In a study conducted with the method of PCR and DNA sequence analysis with isolates obtained from 7 beekeeping regions in Mexico, it was reported that the highest pervalance was 59% and the lowest prevalence was 14%, typical and atypical strains were detected (de Leon-Door et al. 2018).

A wide range of pathogens can cause foulbrood disease. However, European foulbrood is caused by *M. plutonius*. From the isolates taken by cultural methods from the samples obtained from 725 hives in the Northern Marmara region by Borum et al., 37 bacteria were detected by PCR method; however, no *M. plutonius* was found (Borum et al., 2015). The difficulty of culturing in vitro of *M. plutonius* and effective seasonal and regional conditions in its prevalence can change the results (Budge et al., 2010).

While *Mellissococcus plutonius* suspiciously or undoubtedly is available in combs and larvae, noted that they can be found in asymptomatic larvae and adult bees. However, it is observed that they remain enzootic in healthy colonies (Lewkowski and Erler, 2018). European foulbrood disease has been reported to be more severe in bee colonies in the presence of nutrient deficiency and stress (Jyothis and Amritha, 2019). While this study does not consider stress and nutrient deficiency, it is

suggested that researchers should consider these factors in future studies.

European foulbrood disease is caused by *M. plutonius*, and it is a bee larval disease that is widespread worldwide, but its prevalence may vary from country to country and causes serious larval death and extinction of beehives at certain times (Forgsen et al., 2005). It is observed that *M. plutonius* is carried by adult worker bees during feeding and infects the larvae (McKee et al., 2004). Identifying *M. plutonius* in adult bees in this study supports the literature studies.

In conclusion, there is insufficient research on European foulbrood disease in Turkey, and it is believed that more sophisticated epidemiological research and identifying the antibiotic resistance profiles of *M. plutonius* will benefit for eradicating it.

Acknowledgments

Great thanks to Kafkas University of Veterinary Faculty Microbiology Department for all of their supports and contributions.

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******The summary of this study was presented as orally at the 14th National Veterinary Microbiology Congress dated 13-16 October 2020.

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