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Research Article

The Investigation by PCR and Culture Methods of Foulbrood Diseases in Honey Bees in South Marmara Region [1]

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Abstract

American Foulbrood and European Foulbrood diseases of honeybees were examined in 725 beehives from 23 apiaries located in the South Marmara Region of Turkey. We determined that 19 apiaries were infected and the suspected clinical signs of foulbrood diseases were investigated in 102 beehives by PCR and cultural method. Broods and combs from colonies with suspected clinical symptoms of foulbrood diseases were collected and cultured for bacteriological examination. All of the specimens contaminated with bacteriae and 37 species of bacteriae were isolated such as *Stapylococcus epidermidis, Bacillus subtilis, Corynebacterium jeikum, Corynebacterium pseudotuberculosis, Bacillus* spp. All of these bacteria are related to human, animal and environmental origins. In this study, *Paenibacillus larvae* by PCR amplifying the 973-bp region PL1 and PL2 with 1f, *Melissococcus plutonius* amplifying the 973-bp region EFB-F and EFB-R gene were amplified. American Foulbrood causative agent *Paenibacillus larvae* and European Foulbrood causative agent *Melissococcus plutonius* were not detected in any sample examined by PCR and cultural methods. On the other hand, *Paenibacillus alvei* that is a seconder agent to European Foulbrood was found in two samples by cultural methods. In conclusion, the results showed that *P. larvae* and *M. plutonius* are not present in South Marmara Region. In this study, human, animal and environment originated agents were isolated.

Keywords: *Apis mellifera, Honeybee, Foulbrood, PCR*

Güney Marmara Bölgesindeki Bal Arılarının Yavru Çürüklüğü Hastalığı etkenlerinin PZR ve Kültürel Metodlar ile Belirlenmesi

Özet

Güney Marmara Bölgesinde 23 arılıktan 725 kovan Amerikan Yavru Çürüklüğü (AYÇ) ve Avrupa Yavru Çürüklüğü (AvYÇ) hastalığı açısından incelendi. 19 arılıktan AYÇ ve AvYÇ şüpheli ya da infekte 102 kovan PCR ve kültürel metodlar ile incelendi. Klinik olarak yavru çürüklüğü şüpheli kovanlardan petek ve larva örnekleri alınarak bakteriyolojik kültür için kullanıldı. Bütün örnekler 37 tür bakteri izole ve identifiye edildi. Bunlar, *Staphylococcus epidermidis, Bacillus subtilis, Corynebacterium jeikum, Corynebacterium pseudotuberculosis, Bacillus* spp. gibi insan çevre ve hayvan orjinli etkenlerdi. Bu çalışmada, *Paenibacillus larvae* 973 bp'lik PL1 ve PL2, *Melissococcus plutonius*'un 973 bp.'lik EFB-F ve EFB-R gen bölgeleri PCR ile amplifiye edildi. Hiçbir numunede *Paenibacillus larvae* ve *Melissococcus plutonius* üremesi olmadı. AvYÇ'nin sekonder etkeni *Paenibacillus alvei* kültürel yöntemle iki örnekte izole edildi. Bu çalışmada, Güney Marmara Bölgesinde *P. larvae* ve *M. plutonius* varlığı saptanmadı. Çalışmada yavru çürüklüğü şüpheli kovanlardan insan, çevre ve hayvan orjinli etkenler izole edildi.

Anahtar sözcükler: *Apis mellifera, Bal arısı, Yavru Çürüklüğü, PCR*

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INTRODUCTION

Bacterial, viral and fungal originated brood diseases have been reported [1-3]. The most important diseases of bacterial origin are American Foulbrood (AFB) and European Foulbrood (EFB) in honeybees. AFB is caused by the bacterium *Paenibacillus larvae (P. larvae)*. AFB infection is one of severe bacterial diseases of honeybees and widely contagious. The disease is major problem for many beekeepers and causing great losses to the beekeeping industry. *P. larvae* spores are very highly resistant and could survive in the environment for 30-50 years [2,4-6].

Melissococcus plutonius (M. plutonius) causes EFB, an important disease, that affects brood of honeybees. Several bacteria may be associated with the cases of EFB. Larvae with EFB have a various microflora including *Paenibacillus alvei, Bacterium eurydice, Bacillus laterosporus, Enterococcus faecalis*, and *Paenibacillus apiarius* [7,8].

The accurate laboratory diagnosis of honeybee diseases is very important due to different control and protection methods for bacterial diseases. Traditional diagnosis of bacterial diseases is based on observation of clinical symptoms and microbial cultivation in infected and suspicious materials. Identification of bacterial pathogens of honeybees could be achieved by several methods. There are many sensitive and selective culture media exist to identify the bacterial agents in honeybees. Biochemical characteristics and microscopy of suspected materials are used for routine identification of bacterial agents [8-11].

Molecular techniques have also been developed for the identification of *P. larvae* and *M. plutonius*. PCR-based methods for detecting *P. larvae* have been described by several authors [1,8-13]. The sequences of detection primers were based on 16S rRNA gene of *P. larvae* and *M. plutonius* [8,10,12-14].

The objective of this study was to identify the bacteria that cause the symptoms of foulbrood diseases and the use of the PCR assay, cultivation, in the diagnosis, existence of *P. larvae* and *M. plutonius* from samples.

MATERIAL and METHODS

Larvae were taken from brood combs suspected with clinical signs of foulbrood diseases in apiaries. A total of 725 beehives from 23 apiaries located in South Marmara Region (Bursa, Bilecik, Çanakkale, Balıkesir, Yalova) were examined for AFB and EFB diseases in honey bees *(Table 1)*.

Cultivation of Bacteria

Larval remains from brood comb were collected with a sterile swab for the bacterial isolation and suspended in 5 ml of sterile distilled water [8,10,11,14]. The suspension was incubated at room temperature for 10 min and separated into two samples. Vegetative bacteriae in the first part of samples were killed by incubation at 80°C for 10 min. The second part of samples was not heated. The suspension (200 µl) was used for each medium. The suspensions were inoculated on to different mediums. *Paenibacillus larvae* agar (PLA) was prepared according to Schuch et al.[15]. MYPGP agar was prepared as reported by De Graaf et al.[10]. Brain-heart infusion agar (Oxoid CM375) with thiamin (BHIT) agar with 0.1 mg/L thiamine (Sigma T2645), basal medium for *M. plutonius* based on the original formulation has been described by Bailey [16], and Columbia Agar with 5% Sheep Blood (BD 221263). All culture plates were incubated for 24-72 h at 37ºC in aerobic and microaerophilic environment. All plates were examined on daily basis in order to control the growth of bacterial agents. The isolates were examined by light microscopy following gram and carbol fuchsin stain, catalase test and identified with BBL crystal system (BBL Crystal Enteric/Nonfermenter ID and Gram Positive ID Kits -Becton Dickinson and Company, USA) as previously reported authors [3, 8-11,16,17].

Table 1. *Locations and numbers of hives*
Tablo 1. *Kovan sayıları ve yerleşim bölgeleri*

Locations	Total Numbers of Hives	Numbers of Suspected Hives
Bursa	272	31
Bilecik	65	3
Balıkesir	207	45
Çanakkale	87	5
Yalova	94	18
Total	725	102

Polymerase Chain Reaction (PCR) Assay

We used brood samples for PCR. Primers of PCR were designed on the basis of the 16S rRNA gene of *P. larvae* and selectively amplify a 973-bp amplicon. The PCR primers were used from 16S rRNA gene of *M. plutonius* and 831-bp amplicon. All the negative culture samples for *P. larvae* and *M. plutonius* were subjected to PCR analysis.

The larvae suspected with clinical signs of foulbrood diseases were homogenized in 500 µl PBS. 100 µl homogenate was centrifuged at 14.000 g for 10 min and the obtained pellet was used for DNA isolation. Pellets were suspended in 200 µl enzyme solution (containing of 20 mg lysozyme per ml, 20 mM Tris-HCl in pH 8.0, 2 mM EDTA, and 1.2% Triton) incubated for 37°C for 1 h. After, 25 µl Proteinase K and 200 µl of buffer AL (Qiagen) was added, and the lysates were incubated at 56°C for 30 min and then at 96°C for 5 min. DNA was eluted with 200 µl of elution buffer and stored at -20°C. Bacterial DNA was isolated using the OIAamp DNA minikit (Qiagen) as instructed by the manufacturer [8,10-12,14,17].

Primers used to identify *P. larvae:*

Primer 1: 5'-AAGTCGAGCGGACCTTGTGTTTC -3'

Primer 2: 5-'TCTATCTCAAAACCGGTCAGAGG-3' [10-12].

Primers used to identify *M.plutonius*:

Primer 1: GAAGAGGAGTTAAAAGGCGC

Primer 2: TTATCTCTAAGGCGTTCAAAGG [8,13].

Reference strains of *P. larvae* (ATCC 9545 and NRRL B 3555) and reference strains of *M. plutonius* (ATCC 35311 and NCDO 2443) were used as positive control. Deionized water was used as a negative control.

P. larvae PCR protocol were performed with final volume of 50 µl PCR mixture contained; 5 µl template DNA, 5 µl PCR buffer (containing 2 mM $MgCl_2$), 10 nM of each dNTP, 50 pmol forward and reverse primer, 2 U *Taq DNA polymerase* enzyme (Fermentas).

PCR was performed at 95°C for 15 min followed by 40 cycles of denaturation at 93°C for 1 min, at 55°C for 30 s, and extension at 72°C for 1 min and final extension at 72°C for 5 min. PCR amplified products were examined on agarose (0.8%) gel electrophoresis. DNA amplified by PCR was stained with ethidium bromide and visualised with UV transilluminator [10-12,14,17,18].

M. plutonius PCR protocols were done with 50 µl reaction comprising; 4 mM $MgCl_2$, 200 µM of each dNTP, 100 ng of primers, 5 µl of 10 x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3 µg of *Taq DNA polymerase*.

PCR conditions were performed with initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 6°C for 15 s, primer extension at 72°C for 60 s and final extension cycle at 72°C for 5 min [8,13,19,20]. PCR products were analysed by agarose gel and stained with ethidium bromide. The amplification was performed in a Perkin-Elmer Gene Amp PCR System 2400 Thermocycler.

The amplicons were stored at +4°C until electrophoresis was carried out. Samples of the amplicons (5 µl) were electrophoresed on a agarose gel in Tris-boric acid-EDTA (TBE) gel at 75 V for 1 h. The gel was stained with ethidium bromide (0.5 µg/ml). The PCR products were visualized on a UV light. The sizes of bands were determined by comparing to a standard 100 bp DNA marker (Fermentas). *P. larvae* (ATCC 9545 and NRRL B 3555) and of *M. plutonius* (ATCC 35311 and NCDO 2443) were used as positive control.

RESULTS

After incubation, colonies with different morphologies were Gram and Carbol fuchsin stained and identified by CRYSTAL System (Becton Dickinson, Aalst, Belgium). All of the samples contaminated with bacteriae (100%).

The organisms were isolated in pure culture from 64 samples (62.74%), and isolated in mixed culture from 38 samples (37.25%). Thirty-seven species of bacteriae were isolated from 102 hives *(Table 2)*.

AFB causative agent, *P. larvae,* and EFB primer agent, *M. plutonius,* were not detected in any of the samples. However, *Paenibacillus alvei* was isolated and identified in two samples.

All AFB and EFB suspected samples were negative in PCR. Standard strains were found to be positive in PCR. The microorganisms identified belong to human, animal and environmental origins. All samples showed the clinical findings consistent with AFB and EFB.

Table 2. *Identified bacterial species.*
Tablo 2. *İdentifiye edilen bakteri türleri*

Bacteriae Species	**Strain Number Were Isolated**
Bacillus subtilis	25 (67.56%)
Corynebacterium jeikeium	19 (51.35%)
Staphylococcus epidermidis *Bacillus brevis*	10 (27.02%)
Corynebacterium aquaticum *Bacillus spp.*	9 (24.32%)
Corynebacterium pseudodiphteriticum *Corynebacterium pseudotuberculosis*	8 (21.62%)
Corynebacterium bovis *Staphylococcus pasteuri*	5 (13.51%)
Staphylococcus saprophticus, *Enterococcus faecalis* *Bacillus cereus*	4 (10.81%)
Bacillus pumilus *Aerococcus urinae* *Corynebacterium renale group* *Lactococcus lactis ssp. Cremoris* *Bacillus licheniformis*	3 (8.10%)
Staphylococcus simulans *Gemella morbillorum* *P. alvei*	2 (5.40%)
Staphylococcus warneri *Staphylococcus capitis ssp. capitis* *Alloicoccus otitidis* *Bacillus circulans* *Corynebacterium striatum* *Micrococcus luteus* *Rhodococcus equi* *Sphingomonas paucimobilis* *Corynebacterium spp.* *Providencia stuartii* *Escherichia coli* *Staphylococcus aureus* *Hafnia aluci* *Morganella morgani* *Acinetobacter iwoffii* *Klebsiella oxytoca*	1 (2.70%)

DISCUSSION

American foulbrood is the most dangerous and contagious of the infectious diseases in bees.

Diagnosing of AFB and EFB is time consuming, expensive and has difficult isolation and identification procedures concerning *P. larvae* and *M. plutonius*. PCR is rapid, easy and reliable method for *P. larvae* and *M. plutonius*. PCR is very important tool for diagnosing bee diseases.

In this study, cultural method and PCR assay were tested and existence of *P. larvae* and *M. plutonius* from samples were detected.

Different *P. larvae* and *M. plutonius* identification rates have been reported. Garrido-Bailón et al.[1] reported 1.6% prevalance of *P. larvae* and 0.5% *M. plutonius* in the honey bees. McKee et al.[20] detected *M. plutonius* 27.5% of these samples. Kılıç et al.[17] have identified *P. larvae* in 7% of the samples by the culture growth method and in 8% of the samples by the PCR method. Şimşek and Kalender [22] have isolated *P. larvae* in 32 samples out of 335 (9.55%) in Turkey. Ozakın et al.[3] did not detect AFB and EFB causative agents in Bursa, Turkey. *Paenibacillus alvei*, a seconder agent of EFB, was detected only in two samples. In this study, we did not isolate *P. larvae* and *M. plutonius* by cultivation in suspected larvae of South Marmara Region. Secondary factor of EFB, *P. alvei* were detected in only two samples.

In the study of Govan et al.[12] they used 16S rRNA gene of *P. larvae,* then, selectively amplified a 973-bp amplicon and revealed that this amplicon had high sensitivity. Dobbelaera et al.[21] reported that they used technique to detect *P. larvae* in the DNA extracts obtained from larvae remains infected with American foulbrood. Kılıç et al.[17] reported Af 6 and Af 7 primer pair is a highly sensitive primer pairs. Govan et al.[13] reported that PCR technique is a rapid and reliable method for identifying *M. plutonius* directly from diseased bee larvae. Djordjevic et al.[19] reported that MP1 and MP2 have been shown to be specific for *M. plutonius*. The detection of *M. plutonius* in larvae, in healthy and diseased hives by PCR provides a specific and sensitive method for epidemiological studies in EFB [13,20].

PCR technique is a quick and reliable method for the identification of *P. larvae* and *M. plutonius* directly from larvae samples. PCR procedure of takes approximately 24 hours, cultivation and identification of *P. larvae* and *M. plutonius* may take 3-7 days. The diseases of AFB and EFB cause serious colony losses, thus the short-term diagnosis would prevent spread of these diseases as well as the losses of beekeepers. Therefore, PCR is highly reliable and quick test in the diagnosis of AFB and EFB.

In this study, suspected samples were examined with cultural and PCR methods. In both methods, *P. larvae* and *M. plutonius* were detected as negative. Secondary agent of EFB, *Paenibacillus alvei* was detected as positive by cultural method.

We identified bacteriae related to human, animal and environmental origins. All hives showed similar clinical symptoms consistent with AFB and EFB. Minor brood diseases are less serious than foulbrood diseases. However, these agents are extremely contagious [2]. Clinical signs are similar. Thus, it is difficult to distinguish AFB and EFB. Beekeepers must recognise these signs, and distinguish them from foulbrood diseases. If beekeepers pay attention to basic hygienic practises, it is likely to prevent diseases. Lack of hygiene can cause serious losses. Therefore, accurate diagnosis of the foulbrood diseases prevents spreading diseases and consequent economic losses [23].

In conclusion, the results showed that *P. larvae* and *M.plutonius* are not present in South Marmara Region. Secondary agent of EFB, *Paenibacillus alvei* were detected in only two samples. AFB and EFB causative agents were not detected by cultural and PCR methods. In this study, human, animal and environment originated agents were isolated.

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