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RESEARCH ARTICLE

Detection of American Foulbrood Disease *(Paenibacillus larvae)* By the PCR and Culture [1]

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Summary

American foulbrood (AFB) caused by *Paenibacillus larvae* is a bacterial disease in honey bee larvae that is observed worldwide. The aim of this study was to detect *P. larvae* in honey and beeswax samples from the suspected hives by direct PCR and culture growth. AF6 and AF7, the most sensitive pair of primers, were used to identify the DNA of *P. larvae.* A total of 100 suspected honey and beeswax samples collected from 25 different enterprises were examined. *P. larvae* were identified in 7 samples out of 8 by the PCR positive in total. Eight suspected larvae of honeybees were found positive by PCR. A suspected culture-negative sample was found positive through evaluation with PCR. One beeswax sample and seven honey samples were detected as positive for the larvae in the direct PCR detection.

Keywords: *American foulbrood, Culture, PCR, Honey bee*

Amerikan Yavru Çürüklüğü Hastalığı Etkeni *(Paenibacillus larvae)*'nin PCR ve Kültür ile Teşhisi

Özet

Amerikan yavru çürüklüğü dünyanın her yerinde bal arılarının larvalarında görülen ve *Paenibacillus larvae* tarafından meydana getirilen bakteriyel bir hastalıktır. Bu araştırmada *P. larvae*'nin hastalıktan şüpheli kovanlardan alınan bal ve balmumu örneklerinde kültür ve direk PZR metodu ile teşhis edilmesi amaçlandı. *P. larvae* DNA'sını tespit etmek amacıyla en duyarlı AF6 ve AF7 primer çifti kullanıldı. Bu amaçla, hastalıktan şüphe edilen 25 farklı işletmeden alınan 100 adet bal ve bal mumu örneği incelendi. PZR pozitif 8 örneğin 7'sinde kültür yöntemi ile *P. larvae* teşhisi yapıldı. Sekiz adet balarısı larvası PZR ile pozitif bulundu. Kültür negatif olan bir şüpheli örnek PZR ile pozitif bulunmuştur. Direkt bal ve balmumu PZR denemelerinde 1 tane balmumu, 7 adet bal örneğinde pozitiflik saptandı.

Anahtar sözcükler: *Amerikan Yavru Çürüklüğü, Kültür, PZR, Bal arısı*

INTRODUCTION

A bacterial bee disease, American foulbrood, is a disease that leads to infection in the larvae of honey bees, causing them to die and rot. The disease is caused by *P. larvae* which exist in spore form in the larvae of *Apis mellifera* and other *Apis* species. They are spread by contaminated bees, hives, beekeeping equipment, pollens, and honey [1,2] .

American foulbrood can be easily be diagnosed symptomatically due owing to its typical disease symptoms. Although clinical findings are important in the diagnosis of the disease, exact diagnosis requires obtaining pathological material and conducting laboratory examinations [3,4] .

Conclusive diagnosis is made in the laboratory through bacterioscopy, culture growth and biochemical tests, as well as the holst milk test and the methods using

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immunofluorescence Nevertheless, cross-reactions might be detected with *Paenibacillus alvei* in immunofluorescence [1,5-8]. The method of identification from *P. larvae* culture has been recently developed [9]. However, the information about the direct use of this method in clinical samples is scarce.

The progress of the disease can be stopped by the use of drugs in the early stages of the disease before it infects the entire colony. It has been reported that the infected hives should be removed to prevent the spread of the disease and appropriate antibiotics should be administered along with vitamin syrups to strengthen the bees [8,10]. The bacteria gradually develop resistance to the antibiotics that are used to treat the disease. Therefore, it has been recommended to use antibiotics only when the disease is identified and not to apply antibiotics to the colonies for preventive purposes [11].

In the present study the aim was to investigate American foulbrood in the beekeeping enterprises in the villages in the central district and some other districts of Elazig and the use of the PCR assay in the diagnosis of AFB from clinical materials.

MATERIAL and METHODS

Collection of the Samples

A total of 100 suspected honey, beeswax and the honeybee larvae samples collected from 25 different enterprises. In this study, a 10 cm X 10 cm piece of honeycomb was collected with the honey and the combs with larvae from the hives that were suspected of the disease and were transported to the laboratory in cardboard boxes.

Bacteriological Analysis

A piece of comb containing three or five larvae was selected from the study samples and placed in a screw-lid tube, to which 5 ml sterile water was added and it was made homogenous by stirring with a sterile rod. The suspension was kept at room temperature for ten minutes and placed in the water bath at 80°C for 10-15 minutes to kill the non-sporing bacteria. Following the cooling down of the suspension, the tubes were stirred in vortex and the mixture was cultured in brain heart infusion (BHI) and blood agar (containing 6 µg/ml nalidixic acid and 7% sheep blood with citrate) in order to obtain a pure growth of *P. larvae*. The petri dishes were incubated for 48-72 h at 37°C and at the end of the incubation period, the smears prepared by the colonies in the medium were stained with Gram and Nigrosin to examine the vegetative and spore forms of *P. larvae* [7,12-15].

Indole, catalase, voges-proskauer, methyl red and nitrate reduction tests were carried out to identify the biochemical characteristics of the isolated *P. larvae* strains [3].

DNA Isolation

Ten g of each honey sample was suspended in 10 ml sterile distilled water and was later incubated at 95°C for 6 min. Subsequently, 10 ml of this solution was centrifuged at 4.000 g for 30 min and the obtained pellet was used for DNA extraction. The beeswax samples were processed using a similar method, but 1 g samples were used in processing. The larvae exhibiting the clinical symptoms 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 [16].

DNA Extraction and PCR

All the positively identified isolates (by cultural methods) and the negative samples were subjected to PCR analysis. Bacterial pellets (culture, honey, beeswax, larvae) were incubated in 200 µl enzyme solution (20 mg lysozyme, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1.2% Triton) at 37°C for 1 h. Then, 25 µl Proteinase K and 200 µl buffer AL (Qiagen) was added, and the lysates were incubated first at 56°C for 30 min and then at 96 oC 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. To the PCR mixture with a total volume of 50 µl, 5µl 10xPZR buffer (100 mM Tris-HCl, pH 9.0, 500mM KCl, 15mM MgCl2, 1% Triton X-100), 250 µM of each deoxynucleotide triphosphates, 2U Taq DNA polymerase enzyme (Fermentas), 40 pmol of each primer AF 6f (5'- GCA AGT CGA GCG GAC CTT GT-3') and AF 7r (5'-GCA TCG TCG CCT TGG TAA GC -3')16 and 5 µl target DNA were added. The surface of the mixture was covered with 100 µl mineral oil and the PCR reaction was performed in a Touchdown Thermocycler (Hybaid, UK). Amplification was obtained with an initial denaturation step at 95°C for 5 min followed by 40 cycles at 95°C for 20 sec, and at 50°C for 20 sec, and 72°C for 1 min. The final cycle was carried out at 72°C for 7 min. The amplification was performed in a Perkin-Elmer Gene Amp PCR System 2400 Thermocycler. The amplicons were kept at +4°C until electrophoresis was carried out. PCR-amplified DNA was checked on agarose gel electrophoresis. Tris-boric acid-EDTA (TBE) was used as the electrophoresis buffer solution and electrophoresis was performed in a midi gel electrophoresis tank at 75 V for 1 h. Following electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for 30 min and the results were evaluated using an ultraviolet transilluminator. The sizes

of the forming bands were evaluated by comparing them with a standard 100 bp DNA marker (Fermentas) [17]. Gel electrophoresis of the PCR products with a band with a length of 237 bp revealed a positive evaluation in terms of *P. larvae*. *P. larvae* strain NRLL B-3555 was used as a positive control.

RESULTS

Culture Results

In the present study, 100 honey and beeswax samples obtained from 25 different enterprises were examined with a suspicion of the disease. *P. larvae* were identified in 7 honey and beeswax samples. The preparations from the colonies in the medium were stained with Gram and nigrosin; vegetative and spore forms were detected under the microscope. All the isolated strains, were methyl red and nitrate was positive; whereas the indole, catalase, and voges-proskauer tests were negative. Holst milk test was positive and therefore the isolated strains were identified as *P. larvae*.

PCR Results

A 237 bp amplification product was obtained, corresponding to the expected size *(Fig. 1)*. All AFB suspected isolates were positive in PCR. Of the DNA's obtained from the honey and the beeswax samples directly using an available kit, positivity was detected in 1 beeswax sample and 7 honey samples *(Fig. 1)*.

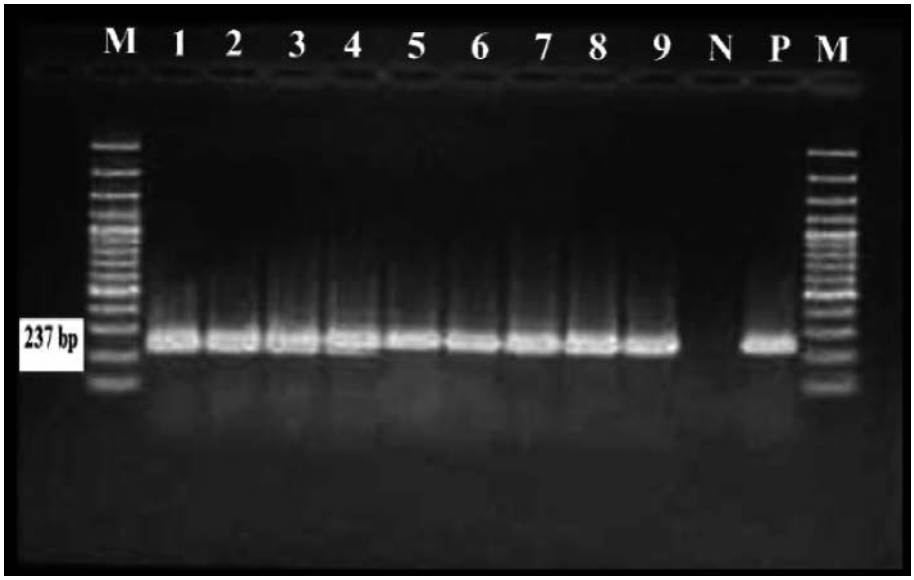

Fig 1. A 1.5% agaorous gel stained with ethidium bromide showing the bands with a length of 237 bp formed as a result of PCR analysis of DNAs obtained from honey and beeswax isolates. **M:** DNA ladder, **N:** Negative control, **P:** Positive control, **1:** Beeswax sample **2, 3, 4, 5, 6, 7, 8, 9:** Positive honey samples

Şekil 1. Bal ve balmumu izolatlarından elde edilen DNA'ların PZR'de analizi sonucu oluşan 237 bp uzunluğundaki bantları gösteren ethidium bromide ile boyanmış %1,5'luk bir agaroz jel. **M:** DNA ladder, **N:** Negatif kontrol, **P:** Pozitif kontrol, **1:** Balmumu örneği, **2,3,4,5,6,7, 8,9:** Pozitif bal örnekleri

DISCUSSION

American foulbrood is the most dangerous and contagious of the infectious diseases in bees. The smell of heated glue in the infected hives and the empty, shrunk, and perforated comb cells observed in the combs with larvae have been reported to be among the specific symptoms of the disease [2-4].

Many researchers [3,4,18] isolated *P. larvae*, from honey directly, while others isolated the agent from comb samples with larvae [19-21]. Although no symptoms of American foulbrood are detected in the hives, it is possible that *P. larvae* spores can be found in honey samples. Since the agent's spore might also infect the hive through marauding bees and contaminated equipment. In such colonies, honey may contain spores even though the disease does not occur and no symptoms are observed. Furthermore, it is argued that the symptoms are suppressed when oxytetracyclin is used against the disease; yet, the agent can still be detected in these hives [22].

Identifying contaminated honey is important in effectively controlling American foulbrood. The identification of *P. larvae* is disputed to be difficult when other *Bacillus* and *Paenibacillus* strains and a few spores are also found in honey samples.

Aydin et al.[3] used 7% defibrinated sheep blood and 6 µg/ml nalidixic citrate added blood agar and brain-heart infusion agar. Akmaz [23] used the brain-heart infusion agar enriched with 0.2 mg/L thiamine hydrochloride and 3 mg/ml nalidixic acid supplemented blood agar in their study. In this study, blood agar with a supplement of 7% defibrinated sheep blood and 3 µg/ml nalidixic acid was used. Several researchers have reported healthy growth in this medium [3,23].

Different *P. larvae* identification rates have been

reported from the suspected colonies. Hornitzky et al.[24] reported 12% of the honey bees, Kauko and Niskanen [25] from Finland have reported 17% of 52 honey samples, Niskanen et al.[26] have identified 6 of 30 samples (20%) and Hornitzky and Clark [4] have identified 12.5-16.5% of the samples in Australia to be detected positive as being infected by *P. larvae*. Aydin et al.[3] have identified 13.76% *P. larvae* from 327 hives and their honey in the market; Akmaz [23] have identified in 65 samples of 300 samples in total (21.6%), while Şimşek [27] have isolated *P. larvae* in 32 samples out of 335 (9.55%) in various studies conducted in Turkey. In this study, *P. larvae* was identified in 7 % of the samples by the culture growth method and in 8% of the samples by the PCR method and the results are in conjunction with other conducted studies.

At the time when this study was conducted, no studies using the PCR method directly on clinical samples for this disease agent were detected. Classical methods for the identification of *P. larvae* in honey samples are more costly and time-consuming in large-scale field surveys. Polymerase chain reaction (PCR) is used in identifying the agent in clinical samples and in epidemiological studies [9]. The PCR technique, one of the molecular techniques, has been used for quick identification of specific DNA of *P. larvae* on bacterial colonies growing in media specific to *P. larvae* [9]. Govan et al.[9] reported that they employed the PCR method to identify *P. larvae* specific DNA among the colonies in a semi-selective medium. Dobbelaera et al.[28] reported that they used this technique to detect *P. larvae* in the DNA extracts obtained from larvae remains infected with American foulbrood and for the identification of the grown bacteria. Recently, Alippi et al.[29] have used the Restriction Fragment Length Polymorphism technique, which allows the distinguishing of the strains of *P. larvae subsp. larvae* from other bacterial species. Bakonyi [16] managed to identify the sensitivity of PCR using different primers with serial dilutions of *P. larvae* isolated from bacterial colonies. The studies have demonstrated that Af 6 and Af 7 primer pair is a highly sensitive primer pair and even a very small amount of nucleic acid amounting from 0.05 Colony-Forming Unit (CFU) of the culture bacteria was sufficient In this study, *P. larvae* DNA isolation was performed using a commercially available kit. AF6 and AF7 primer combination was used for this identification. Studies have demonstrated that AF 6 and AF 7 primer pair identified *P. larvae* in 18 of 23 contaminated honey samples (78.3%) with a high specificity [16]. In the present study *P. larvae* were diagnosed with the culture growth method in 7 samples. PCR was positive in all 8 samples that were suspected of clinical symptoms of AFB. In this study, PCR method was used both to confirm the agents we cultured and isolated as well as to directly identify the agent in honey and beeswax samples. In this study, DNA extraction was performed using a commercially available kit (Qiagen) on direct honey and beeswax samples. Positive results were detected in 1 beeswax (1%) sample and 7 honey samples. Honey was chosen as the target of this study, because it is the vector transporting *P. larvae* spores in the larval guts. During this study PCR amplifications of negative and positive honey samples were repeated twice. In conclusion, PCR can identify the presence of non-germinated spores and for screening for AFB in suspected outbreak areas.

By identifying the disease in samples from commercially available honey and beeswax, producers will be warned about being more careful. Furthermore, it was also aimed to preclude the use of low-quality honey with disease agents as bee food in other enterprises and thus, prevent the spread of the disease. This disease has been declared among the diseases for which filing a notice is obligatory by the Ministry of Agriculture and Rural Affairs and its presence in our country adversely affects our honey and bee products export.

No study has been conducted in Turkey on the identification of *P. larvae* with direct PCR in honey and beeswax samples and through the identification of the disease in honey using PCR, the aim of this study was to put this method in use in routine laboratory examinations. As a result, it was concluded that for the definite diagnosis of AFB, instead of the time consuming and difficult isolation and identification procedures for *P. larvae,* PCR assay which is rapid, easy and specific can be a useful alternative for routine diagnosis.

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