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Original Article
Özgün Araştırma

Frequency of American foulbrood in honeybees: 2015-2020 data of the Veterinary Control Central Research Institute in Türkiye

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Abstract: American foulbrood is one of the most dangerous brood diseases, resulting in huge economic losses for the beekeeping industry on a global scale. The causative agent is Gram-positive, spore-forming *Paenibacillus larvae*. This study aimed to retrospectively evaluate the yearly and seasonal distribution of *P. larvae* isolated from American foulbrood suspected samples sent to the Bacteriological Diagnosis Laboratory of the Veterinary Control Central Research Institute in Türkiye from 2015 to 2020. The presence of *P. larvae* was investigated in the samples obtained from honeycombs with and without larvae and honeybees using conventional methods. Among the total 159 samples examined, *P. larvae* was identified in 49 (30.7%). According to the results, for the 2015-2020 period, the highest number of positive cases was observed in 2017 (8.8%), followed by 2018 (7.5%), 2016 (6.3%), 2015 (5%), and 2019 (3.1%). It was determined that the disease gradually increased from 2015 to 2017 and gradually decreased in the following years. In terms of the seasonal distribution of the positive cases, most were seen in summer (11.9%), followed by spring (9.4%), autumn (8.2%), and winter (1.3%). The data on American foulbrood in Türkiye mostly consist of regional studies, and there is still a need for national epidemiological studies on this subject.

Keywords: American foulbrood, honeybee diseases, *Paenibacillus larvae*.

Bal arılarında Amerikan Yavru Çürüklüğü sıklığı: 2015-2020 Veteriner Kontrol Merkez Araştırma Enstitüsü verileri

Özet: Amerikan Yavru Çürüklüğü, arıcılık sektöründe küresel düzeyde büyük ekonomik kayıplara neden olan, en tehlikeli yavru arı hastalıklarından biridir. Etkeni Gram pozitif, spor oluşturan *Paenibacillus larvae*'dır. Bu çalışmada, 2015-2020 yılları arasında Veteriner Kontrol Merkez Araştırma Enstitüsü Bakteriyolojik Teşhis Laboratuvarı'na gönderilen Amerikan Yavru Çürüklüğü şüpheli örneklerden izole edilen *P. larvae*'nın yıllara ve mevsimlere göre dağılımının retrospektif değerlendirilmesi amaçlandı. *P. larvae*'nın varlığı larvalı petek, petek, arı örneklerinden etken izolasyonu ve identifikasyonu konvansiyonel yöntemler kullanılarak gerçekleştirildi. Çalışmada 159 örnek incelendi ve bunların 49 tanesinde (%30.7) *P. larvae* izole edildi. Elde edilen sonuçlar göre; 2015-2020 yılları arasında en fazla pozitif olgu sayısı sırasıyla 2017 (%8.8), 2018 (%7.5), 2016 (%6.3), 2015 (%5) ve 2019 (%3.1) yıllarında olduğu tespit edildi. Hastalığın 2015-2017 yılları arasında kademeli olarak yükseldiği ve daha sonraki yıllarda kademeli olarak azaldığı saptandı. Mevsimlere göre pozitif olguların dağılımı incelendiğinde; sırasıyla yaz (%11.9), ilkbahar (%9.4), sonbahar (%8.2) ve kış (%1.3) olarak belirlendi. Türkiye'de Amerikan Yavru Çürüklüğü ilgili veriler daha çok bölgesel çalışmalardan oluşmakta ve bu konu ile ilgili ulusal düzeyde yapılacak epidemiyolojik çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: Amerikan Yavru Çürüklüğü, bal arısı hastalıkları, *Paenibacillus larvae*.

Introduction

Bees are the most important pollinators of agricultural production areas and wild flora. Honeybees are social insects that live in a colony and are adapted to collecting pollen and nectar in agricultural areas (İncluding wild plants). Pollinators, such as bees and birds contribute to 35% of the total global crop production and pollinate 87 of 115 important food crops across the world. Three-thirds of crops producing fruit or seeds for human consumption are partially dependent on pollinators (FAO 2020). During the pollination process, the nectar and pollen provided by the plant are collected by honeybees and used as a source of nutrition and colony development, and pollination by honeybees, in turn, contribute to the seed and fruit production of plants. In addition, the nutrients produced by both bees and seeds and fruits are also used in human nutrition. As a result, pollination does not only benefit bees and plants but also animals and humans by helping to

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meet their nutritional needs. Therefore, bees play an important role in maintaining the existence of many species in nature, ensuring the continuity and diversification of plant gene resources, and protecting biological diversity (Korkmaz 2017).

Human's beekeeping activities and interest in honey have a long history. The emergence of honey oozing from the honeycomb drawn on the cave wall in archeology research suggests that the relationship between bees and humans dates back to 700 BC (Anonymous 1; Anonymous 2). In Anatolia, beekeeping culture is one of the oldest agricultural activities with a history of approximately 9,000 years. The first beekeeping laws in the world belong to the Hittite civilization in Anatolia (Çakmak and Seven Çakmak 2016). Türkiye is located at the intersection of three different biogeographic regions, namely Europe-Siberia, Mediterranean, and Iran-Turan, and is home to approximately 12.000 species of plants, one third of which is endemic. The unique geography of Anatolia allows plants to bloom in different regions at different times of the year, making Türkiye a suitable ecological area for beekeeping (TOB 2021).

Honeybees are attacked by numerous pathogens, such as viruses, bacteria, fungi, and parasites that pose a threat to bee health. Due to their vital role in fruit and seed production, factors affecting honeybee health also affect agriculture (Genersch 2010). American foulbrood (AFB) is one of the most dangerous bee diseases, causing economic losses in the beekeeping industry on a global scale (Genersch 2010). The causative bacterium of AFB was first described by White in 1906 in diseased and dead larvae and named *Bacillus larvae* due to the bacillus shape of its vegetative form. The term AFB was coined by Philips because America was the first place where the disease was discovered (Alippi 1999). In the 1990s, as a result of taxonomic re-evaluation with newly developed molecular methods, the AFB agent was named *Paenibacillus* and classified as a separate genus.

The causative agent of AFB, *Paenibacillus larvae subsp. larvae*, is a microaerophilic, Gram-positive, spore-forming bacterium. Infection occurs when honeybee larvae ingest contaminated food and *P. larvae* spores. Especially 12- to 36-hour-old larvae are the most susceptible to the disease. The colony begins to weaken, and abnormal flying patterns and inactivity are seen among bees. The pearly white color of the larvae first changes to light brown, then to dark brown and finally black, taking on a sticky appearance. When the dead larvae turn chocolate-brown, if a matchstick is inserted into the honeycomb and removed, the contents are pulled out in a thread of 2.5-10 cm (Alippi 1999; Genersch 2010; Bodrum 2014; Schiesser 2014; FAO 2020). Spores do not cause infection in adult bees, but adult bees play an important role in the transport of spores. Spores can survive for 60 years in the soil and 33 years in the hive. They are also very resistant to heating, cooling and chemicals, and contaminate both honey and pollen. Burning colonies with AFB symptoms is considered to be the most effective control method to prevent the spread of the disease and is generally compulsory (Genersch 2010; Jatulan et al. 2015; LeBlanc et al. 2015; Bodrum et al. 2016; Özden 2017).

This study aimed to retrospectively evaluate the yearly and seasonal distribution of *P. larvae* isolated from American foulbrood suspected samples sent to the Bacteriological Diagnosis Laboratory of the Veterinary Control Central Research Institute in Türkiye from 2015 to 2020.

Materials and Methods

In this study, data from analysis results of the 159 samples gathered during the period between January 2015 to December 2020, from the AFB-suspected hives and sent to the Bacteriological Diagnosis Laboratory of the Veterinary Control Central Research Institute and subjected to cultural, biochemical and microscopic analyses were used. These samples were from honeycombs with larvae (n=52), honeycombs (n=44), bees (n=62), and honey (n=1). The cases where AFB was detected by analysis (OEI 2016) were evaluated retrospectively.

For the suspected adult bee, larvae and pupa samples, the dead larvae and pupa collected from the wells with sterile swab were suspended in 5-10 ml of sterile water or an isotonic solution (PBS or 0.9% NaCl) in a tube. The suspension was heat-shocked to kill other spore-forming microorganisms (saprophytic contaminants) at 80ºC for 10 minutes or at 95-96ºC for 3-5 minutes in a bain-marie. After the suspension was cooled at room temperature, it was inoculated on to 5% sheep blood Colombia agar (Oxoid, United Kingdom), brain heart infusion agar with thiamine (Oxoid, Basingstoke, Hampshire, England), and nutrient agar (Oxoid, United Kingdom) media. For the honey samples, after honey was diluted by half with sterile water, it was centrifuged at 3,000 g for about 30 minutes. The supernatant was discarded, and the sediment was inoculated onto 55% sheep blood Colombia agar, brain heart infu-

sion agar with thiamine, and nutrient agar media. In order to allow for the disease agent to grow in the media, the Petri dishes were incubated at 37 ± 1°C in an environment with 5% CO_2 for four to seven days, and honeycomb samples for up to 14 days. The developing colonies were evaluated in terms of colony morphology. The colony preparations were stained with the Gram and Nigrosin staining method. As a result of bacterioscopic examinations, the Gram-positive bacilli and zigzag-shaped bacilli in Nigrosin staining (figure 1) were accepted as the typical appearance for *P. larvae*. Biochemical tests used in the identification of bacterial isolates were as follows: Holst milk (+), catalase (-), nitrate (-), nutrient agar (-), indole (-), and Voges-Proskauer (-). As a result of the biochemical tests, *P. larvae* was identified. *P. larvae* ATCC 9545 was used as the positive control strain and *Paenibacillus alvei* as the negative control strain.

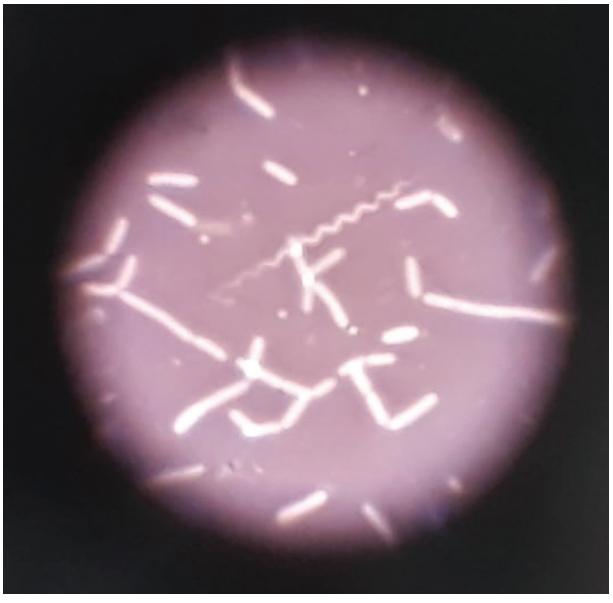

Figure 1. Nigrosin staining

Results

AFB was positive in 49 (30.7%) of the 159 samples examined retrospectively. Table 1 presents the percentages of samples detected to have AFB for the period from 2015 to 2020. The distribution of AFB cases according to the season and sampling method are shown in Tables 2 and 3, respectively.

Table 1. Distribution of American foulbrood cases detected during the 2015-2020 period

Year	Received samples (n/%)	Positive cases (n/%)
2015	29 (18.2)	8 (5)
2016	25 (15.7)	10 (6.3)
2017	35 (22)	14 (8.8)
2018	45 (28.3)	12 (7.5)
2019	22 (13.8)	5 (3.1)
2020	3 (2)	0
Total	159 (100)	49 (30.7)

Table 2. Distribution of American foulbrood cases during the 2015-2020 period according to the season

Season	Received samples (n/%)	Positive cases (n/%)
Winter	10 (6.3)	2 (1.3)
Spring	54 (34)	15 (9.4)
Summer	46 (28.9)	19 (11.9)
Autumn	49 (30.8)	13 (8.2)
Total	159 (100)	49 (30.8)

Table 3. Distribution of American foulbrood cases during the 2015-2020 period according to the sample type

Sample type	Received samples (n/%)	Positive cases (n/%)
Bee	62 (39)	18 (11.3)
Honeycomb with larvae	52 (32.7)	15 (9.4)
Honeycomb	44 (27.7)	15 (9.4)
Honey	1 (0.6)	1 (0.6)
Total	159 (100)	49 (30.7)

Discussion and Conclusion

Beekeeping is an important social and economic activity that has been undertaken in Türkiye. Beekeeping contributes to both plant and animal production. While honeybees collect nectar from flowers, they result in pollination among plants. External factors of physical, chemical, mechanical and biological origin can cause various diseases, colony failure, and extinction in honeybee populations. Honeybees use various defense mechanisms to reduce the damage caused by external factors and prevent them from spreading to the colony. In the last decade, there has been a marked decrease in the number of *Apis mellifera* (honeybee) colonies worldwide.

AFB is accepted as one of the most important factors threatening the health of bee colonies, thus pollination by bees and agricultural production (Genersch 2010). In a study conducted in England

and Wales in 2005, Wilkins et al. (2007) detected 49 AFB cases in 37 apiaries. In a surveillance study of 263 apiaries in Slovenia, Žugelj et al. (2021) reported 506 *P. larvae* isolates from 2017 to 2019. Mráz et al. (2021), screening honeybee pathogens in 250 hives from 50 apiaries in the Czech Republic, determined that AFB disease occurred to a lesser extent and only in urban areas. In anoher study, Gilliard et al. (2008), reported the frequency of the AFB was 83-100% in Switzerland between 2004 and 2005, in 237 bee and 67 honey samples taken from the colonies suspected from the disease. In addition, they reported that this rate was between 22-41% in samples taken from colonies that did not show clinical signs. In an AFB screening study conducted in California, 33.3% of the samples taken from 570 colonies were positive (Eischen et al. 2005). In Germany, the rate of AFB positivity was reported to be 27% (D'Alvise et al. 2019). In a survey study from the Philippines, Cervancia et al. (2013) reported that 46% of 139 apiaries were infected with AFB.

In the current study, 159 samples from AFB-suspected hives that were sent to the Bacteriological Diagnosis Laboratory of the Veterinary Control Central Research Institute between January 2015 and December 2020 were retrospectively analyzed, and it was found that 30.8% were AFB-positive. In other studies conducted in Türkiye, Yalçınkaya et al. (2009) reported the prevalence of AFB disease as 29% in Hatay and Adana provinces, and Özden found (2017) the rate of AFB positivity as 27% in Muğla, Aydın, İzmir, Denizli, Manisa, Kütahya and Uşak provinces. Considering the studies carried out by other researchers in Türkiye using survey, clinical examination or culture analysis methods in apiaries where mobile intensive beekeeping is undertaken, the frequency of the disease is observed to vary between 27% and 29% (Özden 2017). The results of our study are confirmative with the findings reported by previous researchers.

Türkiye has the largest share in world in honey production, with a rate of up to 90%. According to the FAO statistics, following China, Türkiye had the second highest honey production in 2019. In addition, Türkiye ranks first in Europe in terms of the number of colonies and honey production (TOB 2021). Its honey production per hive is 12.7 kg, placing Türkiye in the 23rd position in the world (Anonymous 1). Türkiye ranks third in terms of the total number of hives, but the current number of hives and the amount of honey production are not consistent with each other. The low honey yield per colony in Türkiye shows the potential of honey plants is not fully used in the country, and the yield levels of honey bee populations are not sufficient. We think that one of the reasons for this low productivity may be the effect of AFB, which was seen at a rate of 30.7% in our study.

The strength of the colonies is directly proportional to the number of healthy worker bees. As a result of brood deaths caused by AFB, the number of adult worker bees decreases and colonies weaken. The decrease in the number of worker bees negatively affects the production of all other bee products, especially honey. In order to control AFB, the disease should be diagnosed at an early stage. Beekeepers should carefully examine bee colonies and always be alert to the disease. Although clinical findings are important in the diagnosis of AFB, it is also necessary to take a pathological sample and conduct a laboratory test for a definitive diagnosis.

It is important to collect samples at the right time and by experts in AFB disease to reveal the true prevalence of the disease. In our study, when the distribution of AFB frequency by years was examined, it was found that the highest positive case rates were in 2017 (8.8%) and 2018 (7.5%). The fact that the sampling process was carried out by our expert personnel in these years confirms the above-mentioned argument.

The results of this study indicate that while most of the AFB-suspected samples were sent to the laboratory in spring and autumn, the highest number of positive cases was detected in summer season at a rate of 11.9%. These findings are consistent with the results of previous studies reporting that AFB was more common in summer (Jacques et al. 2017; Morawetz et al. 2019). The reason why AFB is frequently observed in summer can be associated with parasitic mite infestation, queen bee problems, weak colonies due to drought, and increased susceptibility to diseases.

In this study, according to the sampling method the percentages of AFB isolation were as follows; 11,3% bee, 9,4% honeycomb with larvae and honeycomb samples. The high positive AFB rates in bee is an important finding because adult bee has an important role in spreading the infection.

AFB is a notifiable disease according to the Regulation on Notifiable Animal Diseases and Notification, published under the Turkish Law on Veterinary Services, Plant Health, Food and Feed, numbered 5996. As per the Regulations on the Protection and Fight Against the American Foulbrood Disease of Honeybees, after all bees have returned

to the hive in the evening, the hive entrance hole must be closed and bees should be killed by applying calcium cyanide, ethyl acetate or any insecticide. Then, all adult bees, larvae, honey and honeycombs of the killed colony must be destroyed by burning. Antibiotics are widely used as prophylactic treatment in many countries, including the USA and Canada. However, antibiotic therapy is not a sustainable strategy because it suppresses the symptoms of AFB and does not affect the bacterial spores that cause the spread of the disease. Antibiotic use has been prohibited in most countries due to the harmful effects of antibiotic residues in honey and other bee products, shortening of larval viability and adult lifespan in colonies, and emergence of antibiotic-resistant bacteria (Schiesser 2014; Locke et al. 2019). A more sustainable and effective approach to AFB in the long term is the development of disease-resistant honeybee lines and the use of natural strategies such as plant extracts, propolis, probiotics (*B. thuringiensis* as a biological control), and phage therapy as an alternative treatment (Schiesser 2014; Jończyk-Matysiak et al. 2021).

Bacteriological Diagnosis Laboratory of the Veterinary Control Central Research Institute receives samples from its areas of responsibility and other veterinary control institutes, significant data were obtained to evaluate the prevalence of the disease in samples collected from a very wide geographical area. Epidemiological data on AFB disease in Türkiye are mostly obtained from regional studies. In order to reach clear data on this subject, there is a need to conduct surveillance studies at the national level.

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Ethical Statement: In this study, data obtained from the routine bacteriological diagnosis activities of Bacteriological Diagnostic Laboratory of Veterinary Control Central Research Institute were retrospectively evaluated. Ethical approval was not required for the study.

The study was submitted to the General Directorate of Food Control of the Ministry of Agriculture and Forestry of the Republic of Turkey and necessary permissions were obtained (04.02.2022/E-71037622-824.01.03-4393672).

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