

Detection and Molecular Examination of Pathogens in Honey and Bees in the Northern Marmara Region, Turkey ^[1]

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Abstract

Honey, which has many positive health effects, is fondly consumed in our country and in the world. Although honey is considered to be a micro-organism-free food because of its antimicrobial and bacteriostatic effects, studies refute this idea. In addition to primary contamination, personnel, tools and equipment used in beekeeping and honey production is a potential source of secondary contamination. In addition, honey, which can carry many microorganisms as a result of cross-contamination, is among the important foods and can threaten public health. Therefore, it is thought that screening of pathogens that may be present in honey would contribute to the studies. Due to the geographical location, the diversity of climate and vegetation, Turkey is located in the upper row of honey producing countries. In this study, 900 samples examined in Kırklareli province in Northern Marmara Region. Kırklareli region has been chosen as the research area since it is considered as an important province in honey production and is a border province located in the industrial zone. The aim of the study was to investigate the presence of parasitological, bacterial, fungal and viral parameters which are important for the quality of the consumer, bee, larvae, colony and honey. According to the obtained data, positive results were found in many parameters and statistically significant results were obtained.

Keywords: Honey, Bee, Larvae, Microbial quality, Pathogen

Kuzey Marmara Bölgesindeki Bal ve Arılardaki Patojenlerin Tespiti ve Moleküler İncelenmesi

Öz

Sağlık açısından birçok olumlu etkiye sahip bal ülkemizde ve dünyada severek tüketilmektedir. Antimikrobiyal ve bakteriyostatik etkileri sebebiyle bal mikroorganizma içermeyen bir gıda olarak düşünülmesine rağmen yapılan çalışmalar bunun aksini ispatlamaktadır. Primer kontaminasyonun yanısıra, arıcılık ve bal üretiminde kullanılan araç, gereçler ve personel potansiyel bir sekonder kontaminasyon kaynağıdır. Ayrıca çapraz kontaminasyonlar sonucunda da birçok mikroorganizmanın taşıyıcılığını yapabilen bal halk sağlığını tehdit edebilme potansiyeline sahip önemli gıdalar arasında yer almaktadır. Bu nedenle ballarda bulunabilecek patojenlerin taranmasının literatüre katkı sağlayacağı ve benzer çalışmalara temel oluşturacağı düşünülmüştür. Coğrafi konumu, iklim ve bitki örtüsü çeşitliliği sebebiyle Türkiye bal üretiminde üst sıralarda yer almaktadır. Çalışmamızda, Kuzey Marmara bölgemizde bulunan Kırklareli ilimizdeki 900 adet örneği incelenerek tüketici, arı, larva, koloni ve bal kalitesi açısından önem arz eden parazitolojik, bakteriyel, fungal ve viral parametrelerin varlığının araştırılması amaçlanmıştır. Hem bal üretiminde önemli iller arasında sayılması hem de sanayi bölgesinde yer alan bir sınır ilimiz olması sebebiyle araştırma alanı olarak Kırklareli bölgesi seçilmiştir. Elde edilen veriler doğrultusunda birçok parametrede pozitif sonuçlar bulunmuş ve istatistiksel olarak değerlendirildiğinde anlamlı sonuçlar elde edilmiştir.

Anahtar sözcükler: Bal, Arı, Larva, Mikrobiyal kalite, Patojen



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INTRODUCTION

Turkey, which has 7 different geographical regions with its own unique climate and vegetation, is an important country for honey production. Our country also contains 75% of the honey plant species identified in the world [1,2].

Honey contains fructose (~38%), glucose (~30%), sucrose (~1-2%), other carbohydrates (~12%), various minerals (~0.2%), proteins (~200 mg/100 g), and water (~17%), and is a nutritionally important food source [3]. Honey has been used as a therapeutic agent in ancient times due to its antimicrobial effect as well as health benefits to the consumer [4,5].

In general, honey pH ranges between 3.4-6.1 and water activities range from 0.5-0.6. Osmolarity, pH and hydrogen peroxide activities are considered as important factors that induce antimicrobial effect. The basic principle for this antimicrobial activity is the oxidation of glucose through the enzyme glucose-oxidase, resulting in the appearance of hydrogen peroxide. However, the antimicrobial effect described above can only be generated by successfully diluting the honey, and hydrogen peroxide, which may be sufficient for antimicrobial activity, cannot be produced in sufficient amounts due to the low water activity of the honey under normal conditions [6-8]. Although some honey types contain some phenolic compounds based on residual non-hydrogen peroxide (such as benzoic acid and some flavonoids) and a small amount of pathogenic microorganism is expected in honey due to the compounds in question, minimum hygiene rules and risk for consumer health in honey produced without food safety systems, it is reported that pathogens may be found as a factor [9].

Foodborne pathogens are considered to be an important risk factor for public health in developed and developing countries because of their worldwide spread. In the United States alone, 76 million cases of food poisoning occur every year, 325,000 of these cases are hospitalized, and 5,000 of the hospitalized cases are reported as fatal or deadly cases [10]. Viruses, bacteria, fungi and parasitic mites are the most common disease factors in beekeeping [11]. The fecal-oral route is an important way for these diseases transmission. The agents contaminating bees through water and food can be transmitted to larvae and pupae by infected bees [12]. Another contamination that may occur in honey is secondary contamination caused by secondary sources of contamination such as personnel, tools and equipment.

Serological methods, electron microscopy (EM) and agar gel immunodiffusion (AGID) test are used for the detection of viruses. However, due to the low specificity, low sensitivity of these methods and the inability to detect latent infections, the use of molecular analysis methods has started to increase [11,13]. Although there are many studies with the antimicrobial and bacteriostatic effects of honey in medical literature, the information about the food-borne pathogens

in the initial flora of honey and information about the reproductive profile of these pathogens is limited [8,14].

In this research presence of total mesophilic aerobic bacteria, total coliform bacteria, *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Clostridium botulinum* (*C. botulinum*), *Nosema* spp., *Ascospaera apis* (*A. apis*), *Aspergillus flavus* (*A. flavus*), *Aspergillus fumigatus* (*A. fumigatus*), *Varroa* spp. was investigated using classical methods. Molecular analysis method (Reverse Transcription-Polymerase Chain Reaction-RT-PCR) was used for investigation of Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV) and Sacbrood virus (SBV).

With this investigation, it was aimed to determine if honey bees and honey production threat to public health in Kırklareli, which is an important place for beekeeping in Turkey.

MATERIAL and METHODS

Sample Collection

The bees, honey and larvae samples collected for analysis for the determined microbiological parameters were collected from direct bee colonies according to cold chain standards and transferred to the laboratory. Visually, the hives which were stagnant, weak and showing disease symptoms (walking on flight boards by swarming their abdomen, wings discrete bees, thrown larvae, slimy-diarrhea soil crumbs, dead *Varroa* etc.) were preferred primarily in the sampling procedure. 10x10 cm² honeycomb honey samples (capping or uncapping) were taken. During the collection of adult bee (at least 150 and over from each colony) and larvae samples (10x10 cm² larvae honeycomb from each colony), the samples were collected from the newly dead and live bees. 900 samples were collected from 300 hives belonging to 300 families that provided the main livelihood sources from beekeeping from 9 districts/regions of Kırklareli province in Northern Marmara Region of Turkey.

Microbiological Analysis

Sample Preparation: 10 g of each samples were aseptically taken and homogenized with 90 mL of sterile saline water. Serial decimal dilutions were prepared from initial homogenate in the same sterile diluents.

Total Mesophilic Aerobic Bacteria: Petri dishes including Plate Count Agar (PCA) and sample diluent were incubated at 35°C for 48 h and the counting of the typical colonies was performed at the end of the incubation period [15].

Total Coliform Bacteria: 1 mL aliquots of each sample dilution transferred to petri dishes including Violet Red Bile Agar Petri dishes were incubated at 18-24 h at 35°C and the counting of the typical colonies was performed at the end of the incubation period [16].

Escherichia coli: 1 mL dilution was added to petri dishes including Tryptone Bile X-glucuronide Agar (TBX) and incubated at 44°C for 18-24 h [17]. In addition to TBX agar, a chromogenic medium containing 4-methyl-umbelliferyl-β-D-glucuronide was used for verification.

Staphylococcus aureus: After 1 mL dilution was added to petri dishes including Baird Parker agar (BPA) with 5% egg yolk tellurite emulsion, petri dishes were incubated at 37°C for 24-48 h. DNase agar and coagulase test were used for confirmation [18].

Clostridium botulinum: Samples were inoculated into cooked meat medium (CMM) and Trypticase-peptone-glucose-yeast extract (TPGY) for the enrichment of cultures. After incubation time (5-10 days), cultures were streaked to anaerobic Egg Yolk Agar and incubated at 35°C for about 48 h under anaerobic conditions. For the honey samples isolation, dilution centrifugation and supernatant filtration methods were used and then isolation samples were added to CMM and TPGY [19].

Ascosphaera apis: Samples were cultured on potato dextrose agar (PDA) at 30°C for 5-8 days. After incubation suspected colonies were examined [20].

Aspergillus flavus and Aspergillus fumigatus: Samples were spread on Di-Chloran Rose Bengal Medium, Czapek's Dox Agar Medium and Potato Dextrose Medium and incubated at 25-30°C [21,22].

Nosema spp.: Intestinal specimens of up to 30 adult bees from each colony were homogenized after extraction. After each sample were homogenized, 1 mL homogenate in 1 mL distilled H₂O were counted in a haemocytometer (Neubauer chamber) under microscope for the presence of *Nosema* spp. spores [23,24]. After homogenization of honey samples taken from the same hive, approximately 1 mL honey sample was taken and 1 mL distilled water was added to per sample. Homogenates were placed on Neubauer slide and microscopic examination was performed.

Varroa spp.: In order to demonstrate the presence of *Varroa* to beekeepers in the field practically, powder sugar shake method was used for detection of *Varroa* mites [25]. Bees and powder sugar were placed into a jar and the jar was shaken. After the mixture in the jar was poured onto a white cover, *Varroa* mites were counted. In the laboratory examination, adult bees were put into a bottle, shaken with gasoline and filtered through a double honey strainer. The *Varroas* shaken on white blotter were counted. The probable *Varroas* of bees were also detected by stereo-microscopic analysis. For larval analysis, larvae and honeycomb cells were frozen. After freezing, it was disintegrated and filtered through a double honey strainer. Finally, it was counted after poured it on white blotter paper.

Table 1. RT-PCR primers used for viruses selected in the study

Primer Name	Primer Sequence	Product Size (Bp)
ABPV 1	5'-agccactatgtgctatcgtat-3'	207
ABPV 2	5'-atggtgacctctgtgcatta-3'	207
BQCV 3	5'-gcaagctctccaatgatag-3'	322
BQCV 4	5'-aagattcagccgagtcctta-3'	322
SBV 5	5'-accaaccgattcctcagtag-3'	487
SBV 6	5'-ccttggaaactctgctgtga-3'	487

Molecular Analysis

In our study, the collected samples were investigated for the honey bee viruses. ABPV, BQCV and SBV were analyzed. For the negative control samples, honey samples obtained from the hives belonging to Istanbul University Faculty of Veterinary, Parasitology Department were used. For this purpose, honey bee/honeycomb/pupa samples collected from different hives (preferably dead) were recorded according to the hives from which they were collected and the samples collected from each hive were homogenized separately on the hive basis, but together on the basis of the sample. Viral RNA contents were extracted from homogenates using the Garbensteiner method using purification and extraction kits [26]. The specific primer sets (ABPV; GenBank Accession No. NC_002548, BQCV; GenBank Accession No. AF183905, SBV; GenBank Accession No. NC_002066), which have been previously issued and approved by the reference laboratories, are provided to be commercially designed [13]. The primary sets used in our study are shown in *Table 1*.

Statistical Analysis

The Kendall's tau b correlation coefficient can be used to test whether two variables are statistically interdependent. Values of Tau-b range from -1 to +1 (100% positive association, or perfect agreement). Kendall's tau b correlation coefficient was used to compare the correlations between each of the study variables [27].

RESULTS

In this study, 900 samples were collected from 300 hives. The presence of parasitological, bacterial and viral parameters of the samples which are important for the quality of, bees, larvae, colony and honey were investigated. Samples collected from bees, honeycomb honey and larvae (bee, honey and larvae samples from each hive) were examined in terms of 13 different parameters, 5 bacterial, 3 fungal, 2 parasitological and 3 viral parameters. The results obtained from the study are shown in the tables (*Table 2, 3, 4*) below.

The numbers mentioned in the sections in the table are the number of samples belonging to the related parameter, which are considered as risky for bee and hive health.

Samples that were positive for the analyzed pathogens (in a single colony - diffuse appearance range) were evaluated as risky for bee, honey, larvae and consumer health.

In some beehives, although the agents were isolated, no significant symptoms related to the diseases caused by that agent were observed. It was concluded that all beehive bees could be at risk by evaluating the possibility that the disease may have in hives that do not show symptoms but contain agents.

All the correlations of the bees, larvae and honeycomb honey samples were examined in terms of the microbiological parameters analyzed. Since the viral agents (acute bee paralysis virus, black queen cell virus and Sacbrood virus) were not detected in any of the samples, the parameters were excluded.

DISCUSSION

Honey is a very optimal nutrient for people of all ages, except for the first year after birth [28]. In particular, the nutrients in their contents ensure that both the nutritional value of honey is very high and helps to activate the human immune system against many diseases. Best quality honey in the world is produced in Turkey by reason of having many flower species. Although honey production is increasing in Turkey, it is below the required level in terms of honey export. The most important reason for this imbalance between production and exports is the parameters of microbiological origin which are transmitted to the hives from primary/secondary contamination sources. The fact that most of the bacterial, fungal and parasitic factors analyzed in our study were positive proved that these factors can survive in honey. This contamination indicates that hygienic criteria are not sufficient for honey production.

In addition to honey, bees are also affected by many factors and they are infected with pathogenic, viral and parasitic factors [29]. Pathogens can be transmitted to bees and larvae via other bees, parasitic mite or environmental factors such as beekeeping equipments and air [30]. While the pathogens of bees and larvae causes economic damage, at the same time, they can pose a risk to public health as a result of contamination of honey by disease factors. In our study, when the honey and larvae were examined, the presence of many parameters was detected, and only the viral parameter were not positive. In terms of the examined hives, there is a risk that many bees may get sick or carry these factors both to the larvae and other bees.

When the bacterial analysis results obtained in our study are examined, honey samples which are considered as the highest number of risks for consumer health in terms of total mesophilic aerobic bacteria parameters are collected from Kırklareli Center, Çağlayık and Lüleburgaz regions. The main reason for this situation is the location features of the study region. Pollen, dust, air, industrialization,

incorrect waste management, the digestive tracts of honey bees and flowers are the main source and cause of contamination [31,32]. There is more population, vehicle traffic and waste potential in Kırklareli central region. There is more circulation of people/animals/vehicles/goods on the borders due to the fact that Çağlayık region is on the border with Bulgaria, and Lüleburgaz region is the industrial zone of Kırklareli province. Tysett et al. [33] reported in a study that they isolated species belonging to the family *Bacillus*, *Enterobacter* and *Micrococcus* in all honey samples examined. In our study, as in the study of Tysett et al. [33] incidence values were determined to threaten the health of the consumer. The height of these values is attributed to the rapidly growing genetic modification capabilities of bacteria, the use of unconscious agents and the lack of application in food safety systems.

According to the findings, 90 (30%) of the adult bee samples, 84 (28%) of the larval stages and 54 (19%) of the honey samples were positive for *S. aureus*. In contrast to Packer et al. [34] Dixon [35] reports that *S. aureus* has been destroyed in honey due to the antimicrobial effect of honey. The positivity in honey samples was significantly lower than the bee and larvae samples. One possible reason for this result is the low water activity and pH values of honey. In this study, a positive correlation was found between *E. coli* and *S. aureus* microbiological parameters. These microorganisms, which are transmitted especially as a result of personnel contamination, may be an indicator that the hygiene criteria are not complied sufficiently.

Clostridium botulinum was one of the other bacteria examined in our study. Infant botulism is the most serious disease caused by the consumption of *C. botulinum* agent in honey. When the agents are contaminated the hives, they can form infant botulism in the dead larvae [36,37]. In this study, *C. botulinum* were detected in both bees, larvae and honey samples as in many studies around the world. While a high level of *C. botulinum* was found in a study in Lithuania, it was found in a much lower level in Serbia [38,39].

The most important fungal infection in honey bees is defined as Ascosphaeriosis (Chalkbrood Disease) [40]. It is reported that chalkbrood disease is very common in the world and in our country and seriously damages the production of bee products [41]. Although the findings obtained from our study are lower than the rates stated by Soysal and Gürçan [42], it has been observed that the agent has continued its existence in the region for at least 9 years and threatened hive and bee health. One of the possible reasons is that the agent is resistant to environmental conditions and can produce spore. Another possible reason is the suitability of environmental and hive conditions in terms of the easily reproducible agent in humid environments.

Aspergillus flavus and *Aspergillus fumigatus*, which are the most common agents of stone disease, were also studied.

No meaningful relationship was found between *Aspergillus flavus*, *Aspergillus fumigatus* agents and the other factors, but significant relationships were found in the presence of bees, larvae and honey samples in statistical analyses. Kırpık et al.^[43] reported in their study in the region of the Caucasus plateau that identified *Aspergillus* in the bees intestinal flora in different colonies (including live bee).

In this study, some parasitological parameters were investigated and *Nosema* spp. was one of the parameters evaluated. 56 (18.7%) of the bee samples were positive while none of the honey samples were positive for *Nosema* spp. According to the statistical analysis, no significant differences were found between *Nosema* spp. and the other parameters. There are many studies carried out in different provinces related to *Nosema*, which is very common in our country. Oğuz et al.^[12] determined *Nosema* spp. spores in Van province. In other studies *Nosema* spp. spore were detected in Kırşehir and Ordu Province^[2,44]. Studies in the world show that nosemosis is a serious problem not only in our country but in the whole world^[45]. Although *Nosema* spores cause a serious risk to the honey producer, studies show that spores are reduced as a result of good hygiene practices^[24].

Varroa spp. which was also among the parasitological parameters examined in our study was positive in some bee and larva samples. Studies have shown that Varroosis is unfortunately common in our country and it is known as a dangerous external parasite that lives on the larvae, pupae and adults of the active honey bees, growing without showing any noticeable signs for a long time^[46,47].

Acute Bee Paralysis Virus infection, which can cause paralysis and death in bees, is common in many countries around the world. Although Anido et al.^[48] found ABPV infection in their study, ABPV was not found in any materials in Chen et al.^[49] study. In this study, ABPV was not found in any samples. BQCV and SBV which are so important in beekeeping affect larvae, pupae, adult bee and cause to severe disease and death. When studies on bees and larvae are examined, it is seen that BQCV and SBV exist in the world^[11,49,50]. There was no positive samples in our results.

In conclusion, in this study in the Northern Marmara Region shows that many bacterial, fungal and parasitological agents that are risky for both bee and public health can be transmitted to bees, larvae and honey. In order to prevent this contamination caused by inadequate hygiene and sanitation procedures and improper beekeeping practices, firstly beekeepers must be included in education programs. It is imperative that the producers and the relevant government agencies cooperate in a series of continuous measures. As a result of further studies investigating the existence of different disease factors that have not been studied in this study, methods to dealing with these factors and possible contamination sources can be determined.

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