AGRISCIENCE AND TECHNOLOGY
Armenian National Agrarian University
ԱԳՐՈԳԻՏՈՒԹՅՈՒՆ ԵՎ ՏԵԽՆՈԼՈԳԻԱ АГРОНАУКА И ТЕХНОЛОГИЯ
International Scientific Journal
ISSN 2579-2822

Journal homepage: anau.am/scientific-journal
doi: 10.52276/25792822-2023.2-181

UDC 638.15:619:579.642.11(479.25)

Research of Colibacteriosis in Honey Bees at Aragatsotn Region and Susceptibility of the Detected Pathogen to Antibiotics

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ARTICLE INFO

Keywords:
antibiotic,
bee,
enterobacteria,
microbiology,
susceptibility

ABSTRACT

The research was carried out in the Ashtarak and Avan communities of the Aragatsotn region of the Republic of Armenia. During the research enterobacteria were found in the samples brought from the Ashtarak community. *Salmonella, Escherichia coli*, or *Proteus* diseases were suspected. API 20 E microbial identification test-system was used for identification. As a result of research carried out in 2022, using the above method, *Escherichia coli* was confirmed in bees in the Ashtarak community of the Aragatsotn region. The susceptibility of the pathogen to antibiotics was also checked and it was found out that the most effective antibiotics are *Gentamicyn, Levofloxacin, Streptomycin*, and *Amoxicillin*.

Introduction

Beekeeping is one of the most important branches of agriculture. On the one hand, it stands out for its high profitability, and on the other hand, the bee is the main pollinator of a number of agricultural crops, due to which the seeding and yield of vegetables, fruit trees, and herbs increases (Mkrtchyan and Pepelyan, 2015). Besides, natural honey is known for its high caloric content, nutritional value, and curative-preventive properties (Abrahamyan et al., 2008).

Beekeeping existed in prehistoric times. It developed in several stages, from wild beekeeping to framed beekeeping (Ayvazyan, 2015). Armenians have been beekeepers for centuries. This is documented in "Anabasis" by Xenophon (431-354 BC), "Bibliotheca Historica" by Diodorus Siculus, "History of Armenians" by Moveses Khorenatsi, and "Bee" by V.Yu. Nekrasov, and also "History of Armenians" by Hovhannes Draskhanakerttsi. A part of the honey produced by Armenia in ancient times was exported to Persia and Byzantium. Even in the "Judgment Book" of Mkhitar Gosh and Smbat Sparapet, there is a special law regarding the purchase and sale of beehives (Kotoghyan, et al., 1961).

It is clear that the number of beehives in the world has increased between 2010 and 2021. In 2010, there were 80.65 million beehives in the world, while in 2021 there was 101.62 beehives as per statistician (www.statista.com).

In parallel with the increase in beehives, assessment and application of appropriate medical and pre-treatment measures is necessary.

All living organisms can be attacked by their natural enemies and bees are no exception. Parasites and predatory insects (www.fao.org), as well as diseases, can harm bee colonies. The symptoms observed in bee colonies differ depending on the type of pathogen causing the disease, so the most effective way to confirm the presence of the pathogen is an appropriate laboratory diagnosis (The National Bee Unit, 2017).

Bees`diseases are infectious and non-infectious. Non-infectious diseases are not transmitted from sick bees to healthy bees, as they lack pathogens. Colibacteriosis is an infectious, bacterial disease caused by Escherichia coli. As a rule, weak bees get sick and die. The occurrence of the disease is facilitated by the decline in bee resistance, insufficient feed, temperature, and high humidity (Hakobyan, et al., 2014).

Bee gut microbiota is simpler than that of mammals (Kwong and Moran, 2016). The gut microbiota of worker bees is dominated by 9 bacterial phylotypes, 5 of which are the main ones: *Gilliamella, Snodgrassella, Lactobacillus Firm-4, Lactobacillus Firm-5, Bifidobacterium*, and a number of secondary ones: *Frischella, Bartonella, Commensalibacter or Bombella* (Kešnerová, et al., 2020). Recent studies have shown that the gut microbiome of bees is essential for metabolism, immune function, growth, development, and defense against pathogens (Raymann and Moran, 2018), while disruption of the microbiome can lead to reduced bee adaptability (Zheng, et al., 2018).

Materials and methods

The research was carried out in the Ashtarak and Avan communities of the Aragatsotn region of the Republic of Armenia. It was cloned first in the field, then in the laboratory.

According to the beekeepers of the Ashtarak community, the disease started in April and after about 10 days, the bee's decline was observed. The preliminary examination of newly fallen bees was done by eye observation.

In order to conduct laboratory research, the declined bees were transferred from the communities in a thermal bag to the Microbiology Department of the "Scientific Center for Risks Assessment and Analysis in the Food Safety Area" CJCS of the Republic of Armenia.

For the purpose of the research, newly declined bees, microbial identification test system were used: API 20E, Vortex Mixer, dyes, and Italian-made antibiotics such as *Streptomycin* 10 mg, *Levomycetin (Chloramphenicol)* 30 mg, *Levofloxacin* 5 mg, *Amoxicillin* 10 mg, *Tetracycline* 30 mg, *Gentamicin* 10 mg, *Penicillin-G (Benzylpenicillin)* 10 mg, *Erithromycin* 15 mg, *Vancomycin* 5 mg, *Cephalotin* 30 mg, mortar, saline, ethyl alcohol 96 %, meat peptone agar, bismuth-sulfite- agar, Muller-Hinton agar, etc.

The Research was carried out in the following methods: observation (eye observation) and microbiological (Gram staining, microscopic observation by immersion method, identification test system and disco-diffusion using discs containing antibiotics in agar medium, the study of the formation of the zone of suspension of bacterial growth caused by the phenomenon of diffusion).

In the laboratory, suspensions were prepared from declined bees taken from the Avan and Ashtarak communities. After 15 minutes of extraction, primary injections were performed on meat peptone agar in test tubes and on bismuth-sulfite agar in Petri dishes in 1 ml portion sizes. Test tubes with meat peptone agar were placed in a thermostat at 37 ^{0}C for 48 hours and Petri dishes with bismuth-sulfite agar for 72 hours. After removing the test tubes and Petri dishes from the thermostat they were inspected with the eye.

In order to continue the research, smears were prepared from bacteria taken from test tubes and Petri dishes. They were stained by Gram's method, and observed under a microscope with a 100- magnification lens using the immersion method.

Reseeding was carried out to identify the bacillus found in the smears prepared from samples of bees brought from the Ashtarak community. It was placed in a thermostat at 37 ^{0}C for 24 h, after which 5 ml of physiological solution was added to the test tube to which bacteria from a single colony grown from seeding were added, and shaken using Vortex a Mixer. Then it was adjusted to the McFarland 0.5 standard. According to the accompanying methodology, a diagnosis was made using the API 20E test system for microbe identification.

The susceptibility of the detected pathogen to antibiotics was determined using the disco-diffusion method according to the instructions (Determination of the susceptibility of microorganisms to antibacterial medicines, 2004): Muller-Hinton's agar medium and 10 Italian-made antibiotic discs were used to set up the reaction. A 1 ml portion size of bacterial suspension corresponding to the above-mentioned standard was added to the Muller-Hinton agar medium in Petri dishes. The reaction results were read

after 24 hours. The zone formed around the antibiotic disc was measured with a Circinus. The size of the zone of microbial growth suspension was determined in mm by placing it on the ruler.

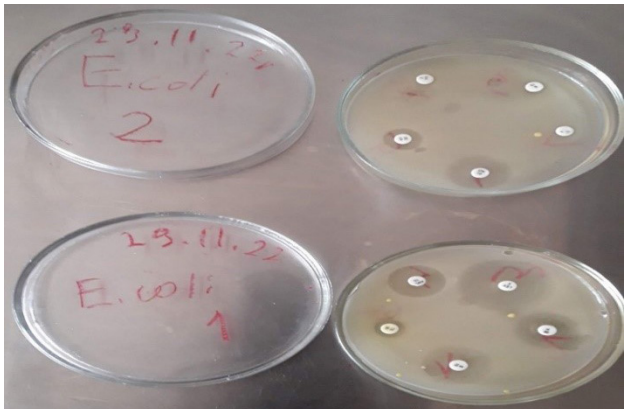
Results and discussions

During field observations, traces of diarrhea were found on honey bread and beehive walls.

During the laboratory research, after removing the thermostat, the test tubes and Petri dishes were examined with the eye. As a result, it was found out that there was expressed white growth in the test tubes of bee samples brought from the Avan community. No bacterial growth was observed in the Petri dishes. In the test tubes of the bee samples brought from the Ashtarak community, expressed colony growth with a light yellowish coloring was observed, and in the Petri dishes, well-established, medium, and large-sized dark brown colonies were noticed, whereupon enterobacteria were suspected (Picure 1).

Picture 1. Growth of brown colonies on bismuth-sulfite agar.

In smears prepared from samples brought from the Avan community, bacteria were absent on bismuth-sulfite agar, and gram-positive stained cocci were found on meat peptone agar. Observations obtained from meat peptone and bismuth-sulfite agars of samples collected from the Ashtarak community revealed 2-3 µm long, gram-negative, non-spore-producing bacteria with concave edges. Microscopic examination showed that these bacteria belong to the Enterobacteria group (*Escherichia coli, Salmonella, Proteus*).



Picture 2. Susceptibility of *Escherichia coli* pathogen to antibiotics.

An identification diagnosis was made using the API 20 E test system for microbe identification, as a result of which *Escherichia coli* 1 was confirmed out of 3 suspected pathogens through the API Web computer program: 99.6 % (very reliable identification).

Bee colibacteriosis was diagnosed through laboratory research. As a result of the microbiological experiments, it was found out that *Escherichia coli* was isolated only from samples brought from the Ashtarak community.

The susceptibility of the *Escherichia coli* pathogen was tested against 10 antibiotics, and the following results were obtained (Picture 2 and Figure).

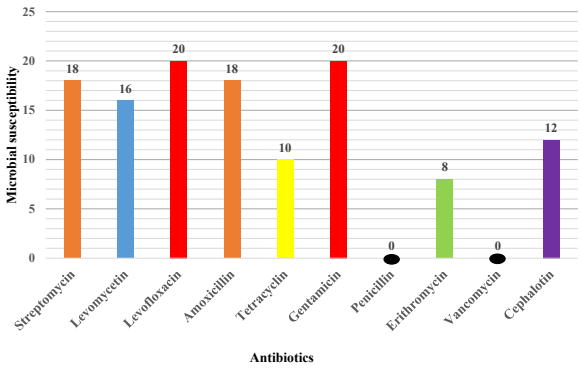


Figure. Susceptibility of the pathogen to antibiotics (*composed by the authors*).

The diagram shows that the following results were obtained when using different antibiotics: *Levofloxacin* and *Gentamicin* with 20 mm inhibition zone, *Streptomycin*, and *Amoxicillin* with 18 mm inhibition zone, *Levomycetin*: 16 mm, *Cephalotin*: 12 mm, *Tetracyclin*: 10 mm, *Erythromycin*: 8 mm and *Penicillin-G* while *Vancomycin* antibiotics did not give results.

As a result of laboratory research on samples from the Avan community of the Aragatsotn region, Escherichia coli, the pathogen of colibacteriosis, was not found. However, gram-positive stained monococci and tetracocci were found in the microscope field of view. But the research is still incomplete.

Conclusion

According to the research results, colibacteriosis (the pathogen: Escherichia coli) disease was detected in the bees brought from the Ashtarak community, while it was not recorded in the bees brought from the Avan community. The decline of bees in the Avan community was not related to colibacteriosis disease and clarification of the reasons needs further research.

Based on the obtained results, it can be concluded that the pathogen is susceptibile to *Gentamicin, Levofloxacin*, *Streptomycin*, and *Amoxicillin* antibiotics. In addition the pathogen shows intermediate resistance to the *Levomycetin* antibiotic and resistance to the remaining antibiotics.

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Accepted on 30.01.2023
Reviewed on 28.03.2023