***Review paper***

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# SIGNIFICANCE OF EUROPEAN FOULBROOD OF HONEY BEES DIAGNOSTICS IN MONTENEGRO

## SUMMARY

The European foulbrood disease of honey bees is a contagious disease primarily of open and rarely of covered bee brood caused by gram positive round bacteria *Melissococcus plutonius*. From the larvae that died of the European foulbrood disease also other bacteria have been isolated: *Enterococcus faecalis*, *Achromobacter euridica*, *Paenibacillus alvei* and *Brevibacillus laterosporus,* but they do not affect the appearance of the disease. These bacteria are involved in the process of degradation of dead larvae. European foulbrood disease is on the list of dangerous infectious diseases of the International Organization of epizootic diseases (OIE). Due to the weakening and deterioration of the affected bee colonies, the disease leads to great losses. At the outbreak of the disease crucial influence have adverse environmental conditions and the mistakes in technology of bee breeding. Symptoms of the disease are visible on the open bee brood; the larvae change color to pale yellow, then dark brown, with semi - solid consistency and then disintegrate. There are no data on the presence of this disease in Montenegro. In order to protect bees from the occurrence European bee brood plague, should be work on the systematic diagnostic and implementation of preventive measures in the apiary. Beekeepers should apply the principles of good beekeeping and good veterinary practice.

**Keywords**: European foulbrood, *Melissococcus plutonius*, Montenegro, bee disease

## INTRODUCTION

European bee brood plague is a contagious disease of primarily uncovered, rarely covered brood caused by gram-positive round bacterium *Melissococcus plutonius (White, 1912), Bailey and Collins, 1983,* fam*. Enterococcacae*

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(formerly *Streptococcus pluton*) (Figure 1). The worker's and drone's litter are susceptible to the disease, as well as the larvae from which the bee queens develop.

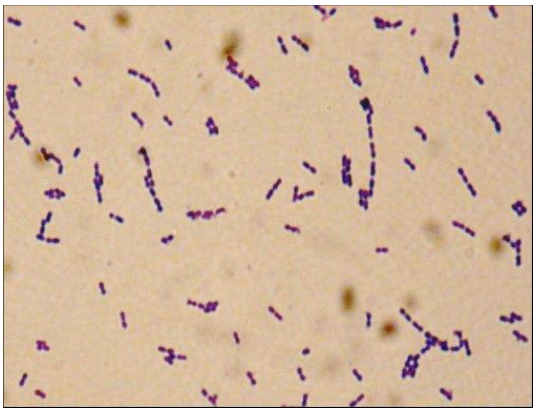

**Figure 1**. *Melissococcus plutonius*, Gram stain (blue-violet cocci arranged singly, in pairs or short chains) (Lundgren and Johansson, Forsgren et al. 2013)

Bacteria *Enterococcus faecalis*, *Achromobacter euridice*, *Paenibacillus alvei* and *Brevibacillus laterosporus* were also isolated from larvae that died from European plague. These bacteria do not affect the occurrence of the disease, but they do affect the process of decomposition of dead larvae (Forsgren, 2010). Although the disease is called the European plague, it is widespread throughout the world and is a growing problem (Mohan Rao et al. 2011). Except in Europe, it is present in North, Central and South America, Asia and Africa. In Europe, it is present in Spain, Switzerland, Sweden, Belgium, Hungary, Great Britain, Italy, Russia, and Belarus. So far, she has not been diagnosed in New Zealand (Bojanić Rašović, 2018c, Anon. 2019, Biová, 2021). It is on the list of dangerous infectious diseases of the International Organization for Epizootics (OIE) and the Rulebook on the classification of infectious animal diseases, the manner of reporting the occurrence or suspicion and reporting of infectious animal diseases (SLCG 92/2017) (Bojanić Rašović, 2018b).

**Basic characteristics of European bee brood plague**

The occurrence of the disease is crucially influenced by unfavorable environmental conditions and errors in beekeeping technology. Climate change, rainy springs and summers, long winters, long retention of bees in the hive, poor grazing, lack of pollen, lack of nectar, a sharp increase in the amount of nectar, a small number of bees that feed brood, nosemosis and other bee diseases, loss of bee queens, microclimatic conditions in the hive (increased humidity), overheating or chilled litter, errors in technology, bee poisoning, stress, etc. are factors that influence the onset of the disease. Low temperatures and poisoning very often precede the appearance of European bee brood plague.

Due to the weakening and decay of diseased bee colonies, the disease leads to large losses. The disease most often occurs in the spring - when bee colonies reach the largest population, but it can also occur in the autumn (Anon. 2015). The European bee brood plague spreads in the same ways as the American bee brood plague. Healthy bee colonies are usually infected through bees that take food through robberies from diseased colonies; the disease can also be transmitted by beekeepers through infected equipment. Within the hive itself, the disease is transmitted by bees that feed the larvae. The causative agent can also be transmitted through the sting of the *Varroa destructor* mite. The manifestation of clinical signs of the disease occurs only in weak and insufficiently fed colonies with weakened immunity and insufficient number of bees that feed the brood. Regardless of the presence of the causative agent of the disease, the disease will not occur if the colony is strong, if there is enough food, if the litter is warm, etc. Larvae 1-2 days old are most often infected. Infection of larvae in the open litter occurs by ingesting food contaminated with *Melissococcus plutonius*. Bacteria multiply quickly in the middle intestine of bees, because they have good conditions and enough food. Depending on the severity of the infection and the amount of food available, diseased larvae will die or survive. The mortality rate of larvae directly depends on the amount of bacteria ingested, the number of bees that feed brood and the amount of ingested food (Lolin, 1991). Infected young larvae become transparent, lose their pearly white color, and due to the reduction of internal pressure, they stretch out and turn towards the aperture of the cell; due to the extension of the larva, the tracheal system is clearly visible (Fig. 2); larvae change color to pale yellow, then dark brown, semi - liquid, gooey consistency, after which they disintegrate (Figure 3).

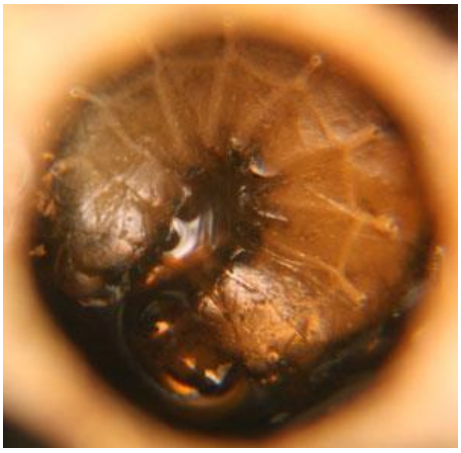

**Figure 2**. Larvae infected with *Melissococcus plutonius* - well visible tracheal system
https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

**Figure 3.** The dead larva is extended towards the aperture of the cell, it is yellowish to dark brown
https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

Larvae usually die at the age of 4-5 days, rarely dying after cell closure. The symptoms of the disease are noticeable on an open litter, unlike the American plague where the symptoms are usually visible on a covered litter. The content of the larvae does not stretch as is characteristic of the American bee plague litter. The stretching test is done by stretching the contents of the dead larvae, usually with the help of a toothpick, a match or a wooden stick (Figure 4).

**Figure 4.** Slightly stretchable content of larvae killed by European plague
https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

If some of the diseased larvae are closed, the appearance of the litter as a whole resembles that of a litter infected with the American plague ("streaked", "scattered" litter). The appearance of a scattered litter is a consequence of death of a certain number of larvae in uncovered and the death or survival of some larvae in covered cells. Holes appear on the lids of cells with diseased or dead larvae - similar to the American plague litter. Holes in the lids appear as a consequence of cleaning the cells and expelling the pathological material by the cleaning bees. Sometimes, the contents of the larva can be weakly extensible - up to 1.5 cm (in the case of American plague, the extensibility is about 2.5 cm) (Bojanić Rašović, 2018a).

Depending on which of the bacteria dominates during the secondary infection, the diseased litter can have a different smell: stale or sour smell, the smell of rotten cheese, rotten fish, the smell of vinegar, but it can often be odorless. Drying of dead larvae creates "scabs" that do not stick to the bottom of the cell and are easily expelled (Figures 5 and 6). (Anon. 2018).

**Figure 5.** Changes on the larva from the moment of infection to its death, decay and drying in the scab (Ruoff, Forsgren et al. 2013)

**Figure 6.** Larvae at different stages of development and disease (Forsgren et al. 2013)

The litter observed as a whole is "streaked" - "scattered" (Figure 7). Litter scattering occurs because some larvae die in uncovered cells, and some die or survive in covered cells. Scattered brood also occurs in some other bee diseases.

**Figure 7.** "Scattered" bee brood infected with European plague https://bee-health.extension.org/european-foulbrood:-a-bacterial-disease-affecting-honey-bee-brood/

At the same time, there are eggs, larvae in different stages and covered cells with dead or healthy dolls on the same honeycomb surface.

Most diseased larvae are detected and removed by cleaning bees. Due to the multiplication of pathogens in the midgut of the larva, infected larvae have a greater need for food. Caregivers bees recognize and expel larvae with excessive food demands. In this way, a strong bee colony can eliminate diseased larvae and keep the European plague under control. Some infected larvae receive enough food to survive and thus prolong the duration of the disease. They pass into the pupal stage, and then into the adult stage. Such puppets and adult forms are stunted - they have a smaller mass compared to the healthy ones and they excrete the pathogen through the feces. Therefore, the disease is most often repeated in treated colonies in the following years, because the causative agent of the disease is constantly present in the hive.

Honeycombs with a diseased litter measuring 10 x 10 cm are taken for laboratory testing. It is best to send honeycombs with freshly dead larvae. Examination of stained microscopic preparations from dead larvae, isolation of the pathogen on nutrient media (Figure 8), as well as molecular diagnostics (PCR) confirm the presence of the pathogen (Ansari et al. 2017).

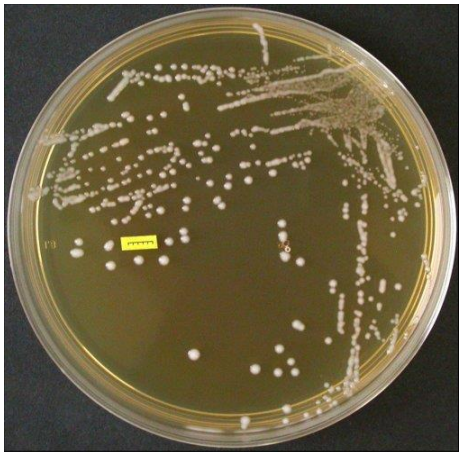

**Figure 8.** Appearance of *Melissococcus plutonius* colonies on basal medium (Lundgren and Johansson, Forsgren et al. 2013)

For microscopic examination, a diluted aqueous suspension is prepared from the contents of the intestine which is transferred to a microscopic slide and mixed with a 5% aqueous solution of nigrosine (Anon. 2018). The aqueous suspension is evenly distributed on 1-2 $cm^2$ of the glass surface, lightly dried on a flame and observed under a microscope. The presence of a large number of lanceolate cocci (having the appearance of a lancet or spear), measuring about 0.5 x 1.0 μm, arranged individually, in groups, pairs or short chains, almost certainly indicates that it is a European plague litter (Figure 1). *Melissococcus plutonius* is a gram positive bacterium that does not produce spores (Figure 1). However, even if it does not create spores, it is quite resistant in the external environment, to the action of physical and chemical factors and drying. Thanks to its resistance, it can survive the winter period on the walls of honeycomb cells, in feces and wax residues at the bottom of the hive, and be a source of infection in the following years as well. On nutrient media under anaerobic conditions for 4 days it grows in the form of small transparent colonies with a diameter of 1 mm (Figure 8). It is very pleomorphic in culture, meaning it changes shape; it can also occur in the form of sticks, especially in cultures that are stored for several weeks. The isolated bacteria can be detected by agglutination in a test tube with the help of antiserum obtained by immunizing rabbits.

**Measures to combat the European bee brood plague**

In order to prevent the appearance of the disease, it is necessary for the bee colonies to warm up in time (especially in the spring), in the absence of pasture to be fed, for the bee queen to be changed every two years, and for the equipment and hives to be disinfected regularly; bee colonies need to be strong and have enough quality food - that is, quality honey. When importing beekeeping equipment, preventive disinfection should be done with a 0.5% solution of

sodium hypochlorite, for 20 minutes or with radiation of 15 kilogray (Anon. 2017a).

When the disease occurs, if the colony is very weak, and the cells are blocked by a large number of dead larvae, the bee colony is destroyed - burned, as in the case of the American plague. If the colonies are on average strong and there is not a large percentage of dead larvae and untreated cells, measures are taken to cure the diseased colony; these are measures that improve the conditions of beekeeping (brood warming, feeding the colony) and measures aimed at destroying and removing pathogens from the hive (disinfection of equipment and accessories, moving the colony to a clean and disinfected hive, etc.) (Bojanić Rašović et al., 2019, Bojanić Rašović, 2019, Bojanić Rašović, 2020a, Bojanić Rašović, 2020b, Bojanić Rašović, 2021a, Bojanić Rašović, 2021b, Bojanić Rašović, 2021c, Bojanić Rašović, 2021d, Bojanić Rašović, 2021e, Bojanić Rašović, 2021 f).

It should be borne in mind that the appearance of the disease in the same bee colonies can occur again in the following years, if the conditions for beekeeping worsen again (Plavša and Pavlović, 2017). Antibiotics have previously been used to treat this disease, but the effects of this therapy are unsatisfactory and harmful. Antibiotic therapy does not lead to the cure of the disease, but only to its transition to another - hidden form. Antibiotics prevent the healing of the bee colony in such a way that antibiotics enable the survival of infected larvae; infected larvae should be allowed to die to be removed by nurse bees. This eliminates the source of the infection. Antibiotic therapy has also led to the emergence of resistance of pathogenic microorganisms to antibiotics and the emergence of fungal and other diseases of bees. Antibiotics leave harmful residues in honey. For all these reasons, antibiotics are banned for use in beekeeping.

## Measures to prevent the occurrence of European bee brood plague in Montenegro

In Montenegro beekeepers have the obligation to report to the veterinary service any suspicion of the occurrence of both this and other dangerous infectious diseases of bees. Measures to diagnose and control infectious diseases are carried out under the supervision of a veterinarian. According to international, as well as national regulations, the European bee brood plague is classified as a dangerous infectious disease and as such it is mandatory for reporting to the International Organization for Epizootics (OIE) (Anon. 2017b, Anon. 2018). The Directorate for Food Safety, Veterinary and Phytosanitary Affairs of Montenegro is obliged to report confirmed cases to the OIE. In Montenegro, there are no data on diagnostic testing of bee colonies for European bee brood plague, nor this disease has been diagnosed in Montenegro. Considering that the disease has been diagnosed in the countries of the immediate and distant environment, in Montenegro the diagnostics and control of beehives for this disease should be carried out. Considering that laboratory diagnostics of European plague is not

performed in Montenegro (no isolation of pathogens is performed), it is necessary to work on the introducing of this microbiological method. Also, in Montenegro there is no defined program of measures to combat this disease, which should include the above measures, as timely warming of bee colonies, providing a sufficient amount of honey for overwintering of bees, supplementing with quality food, replacement of bee queen every other year, regular disinfection of equipment and bee hives, burning of very weak bee colonies suffering from European bee brood plague, etc. In order to prevent the occurrence of infectious diseases of bees, also European bee brood plague**,** beekeepers apply the principles of good beekeeping and good veterinary practice (Grubić, 2018, Rašić, 2018).

## CONCLUSIONS

European bee brood plague is a contagious disease of primarily open, less often closed brood caused by gram-positive round bacterium *Melissococcus plutonius*. Due to the weakening and decay of diseased bee colonies, the disease leads to large losses. The occurrence of the disease is crucially influenced by unfavorable environmental conditions and errors in beekeeping technology. If be colonies suffering from the European plague are very weak, colonies are destroyed. If bee colonies are on average strong, measures are taken to cure the diseased societies. In order to protect the bees of the European bee brood plague in Montenegro, should be done on introducing diagnostics and implementation of preventive measures in the apiary. In order to prevent the occurrence of infectious diseases of bees, beekeepers should apply the principles of good beekeeping and good veterinary practice.

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