IMPORTANCE AND MEASURES OF THE PROTECTION OF HONEY BEES FROM TROPILELOSIS (WITH A SPECIAL FOCUS ON MONTENEGRO)

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Abstract

Tropilelosis is an ectoparasitic disease of bee brood and adult honey bees caused by four types of mites of the genus Tropilaelaps: Tropilaelaps clareae, Tropilaelaps mercedesae, Tropilaelaps koenigerum and Tropilaelaps thaii. Parasites are transmitted by direct contact of bees within one bee colony or contact of bees between bee colonies. During the feeding with hemolymph of bee larvae and puppets, these mites transmit viruses, such as virus of deformed wings (DWV) and black queen cell virus (BQCV). Tropilelosis is on the list of dangerous infectious diseases of the International Organization for Epizootics (OIE). Infestation with this mite causes the death of up to 50% of the bee larvae. It is most commonly found in tropical areas, where throughout the year there is a bee brood that is necessary for the development of parasites. Due to the increasing impact of climate change, uncontrolled imports and the movement of bees and bees’ products, there is a great danger that this parasite will spread to Europe. Poor management in beekeeping, microclimate in beehives and weak bee colonies increase the spread of tropilelosis. In order to prevent the occurrence of this disease in Montenegro, special measures for protecting bees should be defined by adopting new regulations. By removing the bee brood from colony, destruction of all mites in just three days is achieved.

Chemical treatment is not recommended for the fight against tropilelosis. During the first occurrence of tropilelosis, destruction - the burning of infected beehives is recommended.

Key words: Tropilaelaps spp., Tropilelosis, bee diseases, Apis mellifera, Montenegro.

1. Introduction

Tropilelosis is an ectoparasitic disease of bee brood and adult bees caused by four types of mites of the genus Tropilaelaps: Tropilaelaps clareae, Tropilaelaps mercedesae, Tropilaelaps koenigerum and Tropilaelaps thaii. T. clareae and T. mercedesae are the parasites of the European honey bee Apis mellifera, while all four species parasitize on the giant Asian bee Apis dorsata. T. mercedesae is a more present parasite than T. clareae (de Guzman et al., [1], Anon., [2]). Tropilaelaps mites are originally parasites of the honey bees Apis dorsata, Apis laboriosa, and Apis breviligula (Chantawannakul et al., [3]). Tropilelosis is on the list of dangerous infectious diseases of the International Organization for Epizootics (OIE) and the List of Ordinance of the classification of animal diseases, the method of reporting and notification of infectious animal diseases (Anon., [4]). This implies that this disease is compulsory for reporting by the beekeepers to the veterinary service and by the veterinary service to the International Organization for Epizootics. According to Commission Regulation (EC) No. 1398/2003 [5] disease is also compulsory for reporting.

Tropilelosis was first detected in the A. mellifera honey bee in the Philippines in 1961, and then found in the Asian bee A. dorsata in India, Pakistan, Burma, Nepal, Thailand, Borneo. In recent years, it has been one of the biggest problems in beekeeping in Asia (Manić et al., [6]). According to OIE, in the past five years, the disease has been identified in the country of Southeast Asia - Malaysia, the country of Oceania - Papua New Guinea, while in the period from 2014 to 2017, there was a suspicion of this disease in the African country Togo. It is most commonly found in tropical areas, where throughout the year there is a bee brood that is necessary for the development of parasites. Poor man-
agement in beekeeping, microclimate in beehives and weak bee colonies increase the spread of tropilelosis. Due to the increasing influence of climate change, uncontrolled imports and the movement of bees and bee products, there is a great danger that in the coming period this parasite will expand to Europe (Anon., [2], Chantawannakul et al., [3]).

*T. clareae* shows the greatest invasiveness towards *A. mellifera*, and then towards *A. dorsata*. At *A. dorsata* *T. clareae* does not cause significant losses because this species of bee is constantly on the move - after migration it does not develop immediately brood and parasites during this time - until the bee brood develops, cannot survive on adult bees. The most resistant species of bees is *A. cerana*, because it has great self-cleaning ability (Chantawannakul et al., [3]). It should be noted that the presence of this parasite was established in the period April-May 1980 in Serbia; the invaded bumblebee looked like raspberry fruit - on his thorax and abdomen 78 mites were found. At the time when the disease was diagnosed in Serbia, there was no obligation to report it (Manić et al., [6]).

2. Protection of honey bees from tropilelosis in Montenegro

2.1 Morphological and biological characteristics of mites *Tropilaelaps* spp.

These mites are classified in the class *Arachnida*, the subclass *Acari* (*Acarina*), the superorder *Parasitiformes*, order *Mesostigmata*, family *Laelipidae* and genus *Tropilaelaps* (Anderson and Morgan, [7], Anon., [2]). Subclass *Acarina* also include ticks, which are larger than mites; mites are 0.01 - 1 mm in size and ticks can be 10 - 20 mm in size).

*Tropilaelaps* mites are visible to the naked eye - they can be 0.7 to 1 mm in size. The body of tropilelasp is longer than wider, which allows them great mobility on the honeycomb and between the hairs on the body of the bee (Anon., [8]). Species *Tropilaelaps* spp. have a reddish-brown body covered with numerous short spikes. From the ventral side there are four pairs of legs composed of a segments and ending with ambulacrae - membranous leeches with two nails - the claws are turned arched aside. They hold the first pair of legs upright. From the dorsal side they have a dorsal shield, and from the ventral side is an anal plate of an ellipsoidal shape that frames the anal opening of the round shape (Manić et al., [6], Anon., [2]) (Figures 1 and 2).

The length of the body of the mites depends on the species and varies between male and female. *Tropilaelaps koenigerum* is the smallest member of the genus, females are 0.7 mm and males 0.575 mm in length. Females of *T. mercedesae* are between 0.95 to 0.99 mm in length, *T. clareae* 0.87 - 0.885 mm and *T. thaii*
0.89 mm, while the length of males is slightly less - \( T. \text{mercedesae} \ 0.907 - 0.927 \) mm, and \( T. \text{clareae} \ 0.852 - 0.858 \) mm. The length of the male \( T. \text{thail} \) has not yet been determined (Anderson and Roberts, [9]). These mites should be clearly distinguished from other ectoparasites of honey bees, such as the bee louse \( \text{Braula coeca} \) or other mites from the family \( \text{Laelapidae} \) that live in the waste beehives - such as \( \text{Mellitiphis alvearius} \) (Figure 3).

The differences between the \( \text{Tropilaelaps} \) mites and the \( \text{Varroa destructor} \) are determined using a loupe of magnification of 10 x. The body of the varoa is wider than longer (the varoa is 1 - 1.7 mm long, 1.5 to 1.6 mm wide), it slowly moves, while the body of the tropilaelaps is elongated (0.6 - 1mm long, wide 0.4 - 0.5 mm) and move rapidly (Figure 4) (Anon., [2]).

Parasites are transmitted by direct contact of bees within one bee colony or contact of bees between bee colonies (Anon., [10]). Mites can be spread by dividing bee colonies, moving frames with bee brood from one colony to another, moving colonies, illegally buying queen bees and bees. Accidental transfer of swarms to cargo ships, trucks, allow the spread of invasive species to long destinations. It is possible to transfer this parasite to ships coming from tropical Asian countries (de Guzman et al., [1]). The causative agents of tropilaelosis parasitize on the bee brood by feeding the bee larvae and puppets hemolymph (Figure 5), causing serious damage and then the death of the brood (Anon., [8]).

Only 3 - 4% of adult mites are found on adult bees, while the remaining 96 - 97% are found in the bee brood where they are reproduced. The degree of reproduction is proportional to the amount of bee brood in a bee colony. Infection with \( T. \text{mercedesae} \) may be greater than 90%, leading to major economic losses. The mites of the \( \text{Tropilaelaps} \) genus can be seen by visual inspection of bee brood, adult bees or waste at the bottom of the beehive. An unusual appearance of a brood, dead or deformed larvae and puppets, bees with defective wings crawling at the entrance to the hive and the presence of the parasite itself - red brown very moving elongated mites present on the bee brood and adult bees, confirm the disease (Somerville, [11]) (Figure 6).

Changes on the bee brood resemble changes at varroosis (holes on the lids of the brood, scattered brood, changes on larvae) (Woyke, [12]). Perforated lids of cells are the result of cleansing activities by bees' workers, which are ejected infected puppets and young adult bees. During the feeding by hemolymph of bee larvae and puppets these mites transmit viruses, such as virus deformed wings (DWV) and black queen cell virus (BQCV). It has been proven that the DWV virus is multiplied in \( T. \text{mercedesae} \), so these mites are considered a reservoir and biological vector of this virus (de Guzman et al., [1], Anon., [2], Wu et al., [13], Chantawanakul et al., [3]).
The importance of the mite-virus complex has not been fully studied. Some data indicate that the parasite - due to diet with the hemolymph of the bee brood has the greatest effect on the reduction of the immunity of the bee brood. Parasite reduces the concentration of proteins in infested larvae, leading to a weakening of their immune response and the development of viral infection (Khongphinitbunjong et al., [14]). Due to the transmission of viral infection to the bees brood, later in adult bees, deformities occur - deformed abdomen, wings and legs. The mass of diseased bees when they leave the cell is smaller and their lifespan is shorter compared to healthy bees (Dong et al., [15]). Severe infestations lead to the death of the entire bee colony or to their escape. Colonies that fleeing transfers mites to a new location. Infestation with this mite causes the death of up to 50% of the bees larvae (Hosamani et al., [16]). Because of the morphological and physiological deformities and the reduction of bee populations, colonies become vulnerable to infestations with wax moths.

The parasite attacks a drone and bee worker brood, where the drone brood is more sensitive and can be infested to 100%. One cell of the bee brood can infect up to 10 mites. The female parasite lays 3 - 4 eggs on the mature larva of the bee - just before the cell covering (Anon., [17]). Adult females of parasite lay eggs two days after fertilization. Unlike the varoa requiring feeding by hemolymph of honey bee to lay the eggs, the feeding of tropilaelaps mites with hemolymph is not necessary for this purpose. Eggs of Tropilaelaps spp. are most commonly used when bee larvae are in the stage before puppet, then in the stage of larvae rotation, as well as in the bee puppet two days old. Egg laying is rarely occurring in a puppet bee workers older than 1 - 2 days. Unlike a varoa that lays eggs at intervals of 30 hours, T. mercedesae lays eggs faster, every 24 hours (de Guzman et al., [1]).

The overall development of Tropilaelaps spp. from eggs to adult mites lasts for a week (6 - 7 days) and takes place in a covered bee brood. According to some authors, the total development time of mites on the brood of bees’ workers is 6 to 9 days; male developing 24 hours earlier. Development is much shorter than at Varroa destructor, and therefore it is believed that tropilaelosis is a more dangerous ectoparasitosis than varoosis. Before it develops into an adult form, mites pass through three stages of development: larvae, protonymphs and deutonymphs. The egg incubation time is only 0.4 days, and the larvae is 0.6 days; for the development of protonymphs it takes 2 days, and for a deutonymphs 3 days. Immediately before the bees workers left cell, only the late phase - deutonympha was found, whose development ends with the exit of an adult bee. The mites of the genus Tropilaelaps are much more rapidly multiplied than Varroa destructor and thus become dominant in a colony that is infected with both types of parasites. While the varoa creates a major injury, tropilaelaps makes several minor injuries on the body of the developmental forms of bees (de Guzman et al., [1], Chantawannakul et al., [3]). The ability of unmated females’ tropilaelaps mites to lay both males and females and to reproduce without a phore-sy period on adult bees, can be a significant advantage of these mites in relation to varoa mites in Apis mellifera colonies (de Guzman et al., [18]).

Developed adult mites (usually one male and a few females) exit from the cell together with a developed adult bee looking for new hosts. Mites spend a short time outside the cells of the honeycomb (about 1.3 days), while varoa outside the honeycomb cells can live for 13 days. Tropilaelaps mites cannot feed with the hemolymph of adult bees, but it was noticed that the parasite was fed on the soft parts of the bee body - on the membranes around the joints of the wings. It was found that mites can feed with the larvae also in uncovered bee brood. On 4-day old bee larvae (which are constantly added during the experiment) mites can live for 4 weeks, on bee puppets live for 5 days, and on adult bee (without food) for 1 - 3 days. Up to 88% of adult mites die on an adult bee during the second day of their stay (Woyke, [19], Somerville, [11]). The parasite cannot be fed to the adult honey bee with hemolymph because the mouthpiece cannot break through the chitin cuticle of adult bees. Because of that adult bees can live for most two days (according to some authors for a maximum of 7 days) (de Guzman et al., [1]).

2.2 Diagnostics of tropilaelosis

The disease can be detected by a clinical examination of the bee brood and adult bees. The first sign of infestation is the appearance of red-brown elongated mites on a drone and worker bee brood and adult bees. Early diagnosis can be done after the opening of cells with a brood and the finding of immature and mature mites in them. Mites can be detected inside a covered brood using a beekeepers fork, which removes lids cells. The developmental forms of the mites are whitish and almost immobile, and the feed with hemolymph attached the mouthpiece and forelegs for the larval and puppets cuticle (Figure 7).

The infestation intensity is estimated based on the inspection of a certain number of cells with bee brood and expressed as a percentage of the covered brood containing live mites. Drone or worker bee brood are examined by opening 100 - 200 coated cells. The number of mites per bee brood is best tested on a frozen piece of covered honeycomb with a bee brood because of the rapid movement of adult mites on the hive. Examination of waste for the presence of mites is done by placing the adhesive plate at the bottom of the hive.
on which it mites after the fall are stuck. A definitive diagnosis is set in the laboratory by a microscopic examination. The confirmation of the microscopic examination is done by molecular methods, PCR polymerase chain reaction and sequencing (Anon., [2]).

Samples for laboratory testing can be honeycomb with brood, bees, queen bees, drones and waste from the bottoms of the beehive. It is best to take the frames of brood with the bees that come out, as well as the bees brood before closing. A sample of 300 bees from the bee's nest is collected in a glass jar or in plastic bags containing about two tablespoons of powdered sugar, which are then well closed. Bees are collected in a jar by scraping or suctioning with a vacuum. After collecting, the bees are shaking up to the bottom to of the vessel in a layer of 2 - 2.5cm. Instead of powdered sugar, it can be sprayed with ether for two seconds, immersion in 70% ethanol, soapy water or is added 25 g of flour - in order to separate the mites from the bees and prevent their escape. The jar shakes vigorously for 1 - 2 minutes to separate mites from the bees. The bees are then placed on the mesh, mites pass through the mesh into a bright paper or a bright container; bees remain on mesh. Then, the water mist is slightly dispersed over the paper so that the powdered sugar is dissolved in order to accelerate the finding and counting of mites (Anderson and Roberts, [9], Anon., [2]). For the purpose of killing parasites, samples of honeycomb with brood, bees, queen bees and drones are treated with 70% alcohol or freeze before sending to the laboratory. Denatured alcohol should not be used if the molecular diagnostics of the parasite is done. If the parasites arrive at the laboratory live, the samples are placed at -80 °C about 1 hour before the opening of the bag. This procedure immobilizes the mites and can later be stored in 70% alcohol. “Bump method” in the purpose of detection mites in the brood means shaking the honeycomb on white paper - which helps the removal of mites from the brood. It takes frames with a brood containing older puppets - which have eyes of violet color, because the younger brood is more sensitive to shocks - shaking; a small quantity powder of sugar is placed on the white paper sheet that prevents the mites from escaping, then a small amount of water is dispense to dissolve sugar and facilitate the locating and counting of mites; before shaking, removal of the cell lids by scratching is done in order to free the mites from the cells. This latest technique accelerates the detection of mites, especially when the infestation is small (Pettis et al., [20], Anon., [2]). Mites that naturally fall can also be tracked for at least 24 hours using adhesive traps that are placed at the bottom of the panel covered by the mesh. Waste in the hive can be assembled onto a white paper coated with a mixture of petroleum gel and vegetable oil in a ratio of 1 : 1 or commercially available adhesive material (de Guzman et al., [1]). The morphological examination of the mite is done as follows: a drop of lactic acid is placed on the microscope plate, and with the help of very thin tweezers or conifers, the mites are placed in that drop by turning the ventral side of the parasite for observation. Then carefully - if it is possible, without pressure, cover glass over the parasite is laced, so as to avoid creating air bubbles. Tweezers stretch legs mites that are usually hidden - underlined below body. The preparation is viewed under a microscope magnifying 100x, 200x, 400x, to detect differences and details between species. Microscopic preparations can be prepared for storage by previously immersing in Hoyer’s medium (heated aqueous solution of arabian rubber, chloral hydrate and glycerol), then dried for 2 weeks at 50 °C, after which the cover glass is glued to the edge of the nail polish (Anon., [2]).

2.3 Measures of protection of honey bees from tropilaelosis

Removing the bee brood from the colony is an effective measure for the destruction of all mites in three days. This procedure prevents the laying of egg mites,
which reduces the number of offspring of mites that go into adult shape. As the surface of the bee brood decreases, the mortality of the mites increases. Bees cleaners remove the infected bee brood and thus reduce the number of parasites. Since the parasite is completely dependent on the presence of the bee brood, in order to destroy it, the qeen bees are put in a cage for 21 days or more, until the entire brood is hatched and covered; The bee brood then opens, shakes, pulls out and destroys. All mites in three to four days after this treatment disappear (Woyke, [12], Anon., [8], de Guzman et al., [1], Plavša and Pavlović, [21], and Pettis et al., [22]).

Chemical treatment is not recommended for the fight against parasitic mites, despite the fact that the products are easily accessible and easy to use. Pesticides present a dangerous health risk due to product contamination, the accumulation of chemical residues in bee equipment and the development of mites resistance to chemical products. Chemical agents also affect the reproductive condition of the drones and the reproductive performance of the qeen bees. Essential oils are an alternative to the use of chemical agents. Essential oils have an acaricidal effect - they kill mites and reduce their reproduction. They are not toxic and the lesser the problem of developing resistance to them in relation to chemical agents. Essential oil of basil, lemongrass, oregano, lemon and thyme have shown an effective acaricidal effect on tropilaelaps mites (Islam et al., [23]). Formic acid, thymol, combination of thymol and oxalic acid have shown the highest efficiency against tropilaelaps mites. The treatment with fogs of thymol and D-limonen leads to a decrease in the number of these mites. Lemongrass oil (Cymbopogon citratus), applied through a porous ceramic to control the evaporation of this oil, has led to an effective reduction in the number of these mites in Thailand (de Guzman et al., [1]). It has been found that 2% solution of garlic extract reduces the number of this parasite by 72.39%, and does not affect the organoleptic properties of honey. In bee colonies treated with this extract, there was also found a significantly larger number of adult bees, as well as larvae and puppets in the brood (Hosamani et al., [24]). Agents for the treatment of varoa are also effective in controlling tropilaelaps, but the frequency of treatment should be greater than in varoa due to the higher rate of reproduction tropilaelaps mites (Woyke, [25]). Although generally safe, organic acids also have a negative effect on bees. For example, antioxidant effect increases the formation of drones, while thymol and oxalic acid reduce the formation of brood of bee workers. The use of these agents should be interrupted for at least eight weeks before the extracting of honey, in order to avoid contamination. Caution should be observed when handling these acids (de Guzman et al., [1]). The use of preparations that are allowed to treat tropilaelaps can only be carried out with the supervision of a veterinarian.

According to the OIE Terrestrial Animal Health Code [1], for the import of: live qeen bees, bees workers, drones, bees larvae, puppets and honeycombs, importing countries require a certificate that the exporting country is free of tropilaelaps. Qeen bees before importation must be inspected for the presence of parasites and be insulated for 21 days. The consignment should consist only of the qeen bees and the accompanying bees workers without honeycomb. When importing, care should be taken of the uses of apiculture products originating from a country free from tropilaelaps, free of live bees or bee brood, and keeping at least 21 days in bee environment before dispatch, or treated with one of the following procedures: heating at 50 °C 20 minutes, or freezing at -12 °C for at least 24 hours, or fumigation with methyl bromide at a concentration of 48 g/m³ at atmospheric pressure and a temperature of 10 - 15 °C for 24 hours, or 350 Gray radiation. When importing honey, it is necessary to certify that originating from country free from tropilaelaps, filtered through filters with pores smaller than 0.42 mm or treated with one of the following procedures: heating at -50 °C for 20 minutes, freezing at -12 °C or lower temperature - at least 24h, or radiation at 350 Gray. Recommendations for importation of pollen are that originating from country free from tropilaelaps, treated with freezing at -12 °C or lower temperature - at least 24 hours, or 350 Gray radiation, or drying by lyophilization. Recommendations for the import of beeswax or propolis are that originating from country free from tropilaelaps, treated with freezing at -12 °C or lower temperature - at least 24 hours, or by fumigation with methyl bromide at a concentration of 48 g/m³ at atmospheric pressure and at a temperature of 10 - 15 °C for 2h, or irradiation with 350 Gray, or drying by lyophilization (Annon., [10]).

2.3.1 Measures of protection of honey bees from tropilaelaps in Montenegro

According to the Ordinance on measures for the control and eradication of bee diseases (Anon., [26]), and the Program for compulsory measures of animal health protection in 2018 in Montenegro (Anon., [27]), preventive and diagnostic measures are carried out in order to timely detection and control the appearance of infectious diseases of honeybees. The beekeepers are obliged to regularly check all the bee colonies in the apiary, and observed changes that may indicate a bee disease (changes in the behavior of bees, changes in adult bees, changes in the bee brood or death of bees), informed to official veterinarian. However, the aforementioned regulations do not include specific measures to combat tropilaelaps (Bojanić Rašović, [28]). In Montenegro tropilaelaps is not diagnosed or clinical and laboratory diagnosis of this disease is done (Montenegro borders with Bosnia and Herzegovina to the north, Serbia to the east, Albania to the south and...
3. Conclusions

- Tropilelosis is a dangerous parasitic disease of bees and bee brood leading to major economic losses. It usually occurs in tropical areas, where bee brood is present throughout the year without which the parasite cannot develop. However, due to the increasing impact of climate change, uncontrolled imports and the movement of bees and bee products, there is a great danger that this parasite will spread to Europe.

- In order to prevent the occurrence of this disease in Montenegro, special measures for the protection of bees should be defined by adopting new regulations. During the first occurrence of tropilelosis, destruction - the burning of infected hives is recommended.

4. References


