Scientific Works. Series C. Veterinary Medicine. Vol. LXIV (2), 2018
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

IN VITRO TRIAL ON USING AMPROLIUM CLORHIDRAT TO CONTROL *NOSEMA* INFECTION IN HONEY BEES

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Abstract

Nosema spp., a microsporidian parasite (Microspora: Microsporidida), is well known for the negative impact on the bee colony. In areas with temperate climate, the nosemosis's evolution in the apiary is different from season to season. During spring, when in the hive, due to the consumption of honey and bee bread reserves contaminated with spores, the infectious pressure is increasing, the disease worsens, and there is need for a medical intervention. For this, there is an increasing interest for additional products to control this infection. Therefore, this study aimed to test amprolium hydrochloride 20% ($C_{14}H_{19}CIN_4$), a product which has a structure similar to the B1 vitamin (thiamine) towards which it is a competitive antagonist, for controlling Nosema infection in honey bees. The trial was carried out under laboratory conditions and the microclimate parameters have been monitorized. There were used two experimental modules and one control, each module consisting of two batches of bees naturally infected with Nosema spp., with at least 100 bees (122-185) per batch. The batches have been organized according to the current standards, in wooden cages (with the size of 190/150/50 mm) equipped with window, ventilation mesh and feeder. Throughout the trial, the product has proved to control the development of the parasite so as at an initial infection level of approx. 5,750,000 spores/bee, by the end, in the experimental batches no spores of Nosema spp. were detected. Also, there were not signs for adverse effects on bees. However, additional field and laboratory studies are necessary toward to develop an integrated control program over the bees' active season.

Key words*: Nosema; honeybee; amprolium; in vitro trial.*

INTRODUCTION

Microsporidida includes obligatory intracellular parasites forming single-cell spores that infect different hosts; of them, *Nosema* species commonly infect invertebrates, including Hymenopteran insects.

Two species of *Nosema* genus, described as *Nosema apis* and *Nosema ceranae*, cause a serious diseases in honey bees called nosemosis, well-known all over the world for its negative impact on honey bees. Spores of *Nosema* spp. are unicellular and have an extrusive polar tube, long and spiral filament, for the host penetration. Vegetative forms develop in the host, strictly intracellular, in the epithelial cells of the middle intestine, causing digestive dysfunction and metabolic disorders (Aioanei et al., 2011; Mitrea, 2011).

Nosemosis' occurrence in the bee colony, especially in temperate areas shows differences from season to season. This disease is considered to be a major problem during spring, when an imbalance in the host-parasite relationship occurs, with a negative effect on the survival and / or on the production capacity of the honeybee colony (Bailey, 1976; Chioveanu, 2009; Crane, 1975).

Due to the ubiquity of the spores and the precariousness of the host-parasite relationship equilibrium, it is necessary to constantly monitor the disease and to intervene quickly during of critical periods, such as: in spring after winter, in spring and autumn during of

colony unifications and during of the active season, subsequently of the formation of artificial swarms (Chioveanu et al., 2004; Glavinic et al., 2017; Popovici et al., 2012).
At the end of the pause period, in spring, when inside the hive, due to the consumption of honey and beebread contaminated with spores, the infectious pressure increases, the disease becomes acute and treatment is needed. In critical periods, it is necessary to support the bee colony with energy and / or proteic bio-stimulators, supplemented with suppressing drug products (fungi-static or fungicidal ones) against the parasitic stages. In this respect, there is an increasing interest on finding additional products with action against of the *Nosema* microsporidia parasites (Chioveanu et al., 2004; Glavinic et al., 2017)
Given to the anticoccidian spectrum on different species (such as fish, birds, mammals) and starting from the efficiency of Amprolium hydrochloride treatment (icbmv.ro), the aim of the present study was to test under laboratory conditions the potential and efficacy of this product to control *Nosema* infection in honey bees.

MATERIALS AND METHODS

In this study, the product Amprolium hydrochloride 20% solution ($C_{14}H_{19}CIN_4$) – a veterinary antiprotozoal (coccidiostat) that interferes with thiamine metabolism, was assessed. The product has a similar structure to vitamin B1 (thiamine), required by the enzymatic system of glucose metabolism, to which it is a competitive antagonist (http://www.icbmv.ro/).
The biological material used in the study was represented by honey bees (*Apis mellifera*) from an apiary in Prahova county (Valenii de Munte, Romania). Before organizing the experimental trial, a pre-selection study was performed to determine the level of *Nosema* infection by investigating 7 colonies for selection of those colonies appropriate for the experimental study (Table 1).
The level of infection with *Nosema* spores was determined in laboratory using a quantitative method. Briefly described: for each analyzed sample, 60 bee abdomens were macerated in 60 ml of distilled water (ddH_2O). The resulting suspension was centrifuged for 6 minutes at 800 r/min. The sediment was re-suspended, supplementing it to 60 ml with distilled water and examined for presence of *Nosema* spp. spore. From each sample, 10 µl were used to load a Burker-Turk hemocytometer counting chamber and spores were counted (5 aliquots/sample were counted and based on the results the arithmetic mean was determined, obtaining the number of spores/an analyzed unit) (Chioveanu et al., 2009; Ionita and Mitrea, 2013; OIE, 2013).

Table 1. Data on the level of *Nosema* infection (no. of spores) in the investigated bee colonies for selection of the bee batches

Level of *Nosema* infection in the investigated bee colonies (colony ID)	**Number of positive colonies**	**Selection as**
Colonies with 23 spores/unit = 5 750 000 spores/ bee (ID: 724)	1	Experimental colony
Colonies with 1 spore/unit = 250 000 spores/ bee (ID: 759; 778; 625;793)	4	Clinical healthy colonies
Colonies with 5 spores/unit = 1 250 000 spores/bee (ID: 721)	1	Reserve colony
Colonies with 8 spores/ unit = 2 000 000 spores /bee ID: 767)	1	Reserve colony

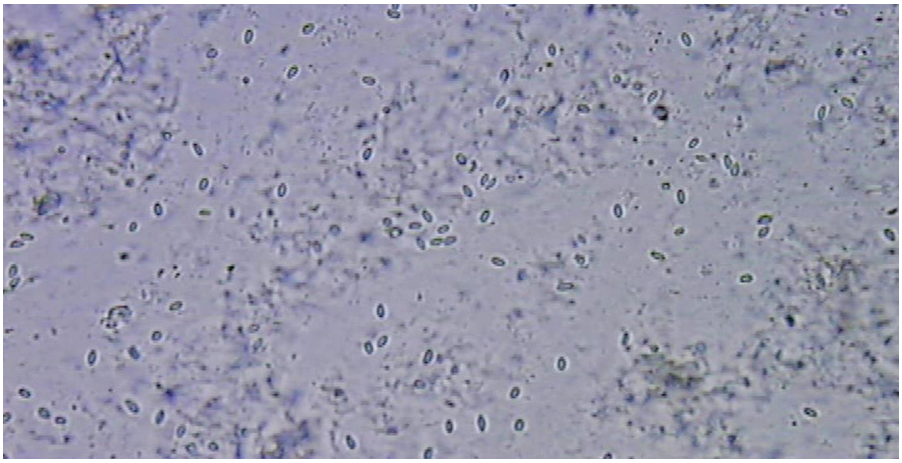

Figure 1. *Nosema* spp. spores in honey bees (direct smear; x 400)

In order to perform „in vitro" trial, two experimental modules were selected from the colony ID-724 where it has been found an infection with *Nosema* spp. of 23 spores/ unit which highlights a level of infection of 5,750,000 spores per bee. Besides of the two experimental modules, in the trial was included also a control module, each module of two batches. The groups of bees were kept in wooden cages (with 190/ 150/ 50mm size) fitted with glass, ventilation mesh and feeding device, with at least 100 bees/cage (Figure 2). The tested bees had at their disposal food such as salubrious crystallized honey.

The trial was conducted in laboratory conditions, in a heated room at a temperature around 28 ºC and 28% humidity (Figure 3).

Figure 2. Bee cage used in the experiment

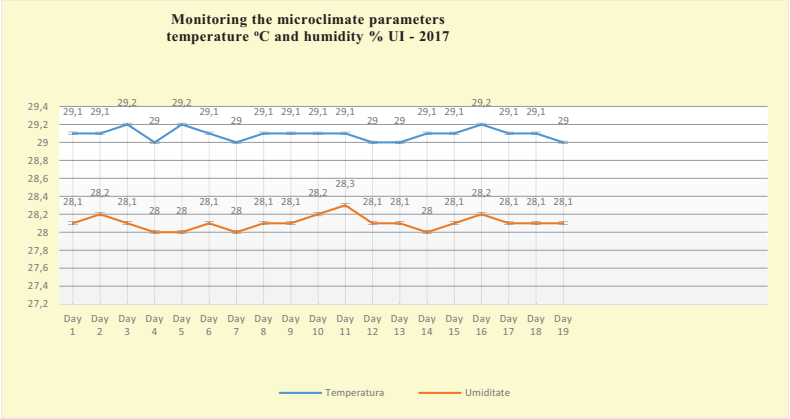

Figure 3. Monitoring the microclimate parameters (temperature and humidity) during the experiment

Amprolium hydrochloride 20% was administrated in 1/1 sugar syrup (60% SU), in an amount of 1g (5ml) per litre. The pH of the sugar syrup was adjusted with food vinegar to 3.5 - 4. The medicated syrup was administrated by spraying on bees, every day, such as: 0.2 ml (200ppm a.s.) (Module 1), respectively 1 ml (1 mg) (Module 2) (Table 2). For the control module, 1 ml of sugar syrup 1/1 (60% SU) was administrated.

During of the trial, daily, there were performed observations regarding the general condition of bees and data on clinical signs and mortality were registered; the dead bees were counted and eliminated from each batch. Samples of dead bees were collected for subsequently laboratory investigations in order to determine the level of infection with *Nosema* spp.

Table 2. Experimental design: the experimental modules and dose of medicated syrup

Module	**Batch**	**Syrup with Amprolium hydrochloride*** (dose)
1. Experimental	1A	0.2 ml (200ppm s.a.)
	1B	0.2 ml (200ppm s.a.)
2. Experimental	2A	1 ml (1 mgr s.a.)
	2B	1 ml (1 mgr s.a.)
3. Control	Control 1	1ml sugar syrup**
	Control 2	1 ml sugar syrup**

*administered only to the experimental modules
** without Amprolium

RESULTS AND DISCUSSIONS

The study regarding the potential of Amprolium hydrochloride product in controlling *Nosema* infection in bees, performed in laboratory conditions over a 19-days period, allowed us to highlight the suppressive effect of the product. Bee colonies from both experimental and control groups were examined daily, registering: the microclimate parameters, clinical status and mortality of bees. The results are presented in Table 3. In this respect, no spores of *Nosema* spp. were found at the end of the trial in the experimental modules (to which the product was administered in different doses), while for bees in the control batches *Nosema* infection was still present and with increased intensivity (from 23 spores / unit = 5,750,000 spores / bees to 31 spores / unit = 7,625,000 spores / bees).

The survival rate of bees in the three modules (the two experimental modules and the control one) was not influenced during of the procedures.

There were no significant differences between the two experimental modules, to which were given different doses; moreover, a 5 times increased dose did not show a negative influence on the analyzed bees. The survival rate of bees in the control batch, under laboratory conditions, during the test registered lower degrees than the survival period of the bees in the two experimental modules. Mortality of bee in the experimental module 1 occurred after 16-19 days, after 16-18 days for the experimental module 2, and after 15 days for the control module, respectively (Table 3).

Table 3. Level of infection with *Nosema* spp. and bee loss (number of dead bees) per day and per module

Day	Batches included into the present study					
	Control Module		Experimentale Module			
	Control 1	Control 2	Batch 1 A	Batch 1 B	Batch 2 A	Batch 2 B
Day 1						
Day 2						
Day 3						
Day 4	2					
Day 5	2	3				
Day 6	9	8		4		
Day 7	8	11	4	6	7	
Day 8	5	10	5	7	9	8
Day 9	8	10	12	13	11	7
Day 10	16	14	14	14	10	12
Day 11	13	19	15	12	14	14
Day 12	23	13	14	13	19	17
Day 13	21	18	15	12	19	19
Day 14	22	17	17	16	17	20
Day 15	13	16	16	17	20	23
Day 16			15	8	18	23
Day 17			18			22
Day 18			18			20
Day 19			22			
Total no of bees	142	139	185	122	144	185
Spores/bee	7625000	7625000	0	0	0	0

From the presented data, the Amprolium hydrochloride 20% has proven to be able to control the development of *Nosema* spp. parasite. In the experimental groups, at the

beginning of the trial, there was recorded an infection rate of 23 spores / unit, which means 5,750,000 spores / bee, while at the end of the experiment, for the experimental batches after administration of Amprolium hydrochloride, no spores of *Nosema* spp. were detected. There were no registered clinical signs or other adverse effects on bees. In the control group, the degree of infection increased from 23 to 31 spores / unit, which represents an infection rate of 7,750,000 spores / bees. In this respect, it is known that at an infection of 36 spores / unit, which highlights 9,000,000 spores/bee, the changes in the intestinal epithelial cells are of irreversible nature (Chioveanu et al., 2009).

Taking into account the manufacturer's recommendations for the use of the product in other species, the therapeutic dose for Amprolium hydrochloride was estimated at 10 mg/kg alive weight (equivalent to 10,000 bees), being administered daily for 7 days in 200 ml syrup, the dose of substance which was able of showing a therapeutic effect.

The therapeutic target for the use of amprolium hydrochloride as a drug is important because the host-parasite's metabolic pathways are common and the product blocks the thiamine absorption of protozoans, preventing the synthesis of carbohydrates. The product does not create resistance problems and no secondary phenomena have been observed. Long-term administration may result in thiamine deficiency (B1 vitamin). Thus, administration of the product should be performed only on a diagnostic basis. Also, due to the very rapid evolution of the disease, the duration of treatment should not last longer than 5-7 days in order to avoid the risk of drug interference with bee metabolism. In case of side effects, the treatment is discontinued and B1 vitamin should be given.

This experimental study was intended to be prospective, for testing and assessing a therapeutic dose. However, additional field studies, in the apiary are necessary. Analyzing the results, Amprolium hydrochloride 20% did not prove to have negative influence for the viability and vitality of bees even at a five times higher concentration than the maximum estimated therapeutic dose. Nonetheless, we admit that products used against nosemosis can stop the disease but without succeeding in the destruction of the spores in the hive. Disease in latent form may re-occur at any time following the emergence of favourable factors. Therefore, use of Amprolium hydrochloride together with effective prevention and control measures can solve this. For this reason, additional field and laboratory studies requires a development of an integrated pest control program on bees' Nosemosis.

CONCLUSIONS

Using the 20% Amprolium hydrochloride product to control nosemosis in bees under laboratory conditions, it was observed that it blocks the multiplication of *Nosema* parasites, preventing the clinical diseases. However, in order to develop an integrated control program for the prevention and control of nosemosis, additional field and laboratory studies are required.

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