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Research article

Nosema ceranae DNA IN HONEY BEE HAEMOLYMPH AND HONEY BEE MITE *Varroa destructor*

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Honey bee mite *Varroa destructor* and microsporidium *Nosema ceranae* are currently considered the most important threats to honey bees and beekeeping. It has been believed that both *N. apis* and *N. ceranae* invade exclusively epithelial cells of the honey bee ventriculus. However, some findings suggest that these microsporidia may infect other tissues of honey bees. There are indications that these pathogens could be found in honey bee haemolymph, as the medium for its distribution to anatomically distant tissues. Knowing that *V. destructor* being an ectoparasitic mite feeds on the *honey bee's haemolymph*, the aim of this study was to investigate if DNA of *Nosema* spp. microsporidia could be found in honey bee haemolymph and in *V. destructor*.

The study was conducted on bee haemolymph and *V. destructor* mites from 44 *Apis mellifera* colonies. From each hive five mite individuals and 10 μL of haemolymph (from 4-5 bees) were used as samples for DNA isolation and PCR detection of *Nosema* spp.

The DNA of *N. ceranae* was confirmed in 61.36% of *V. destructor* mites and 68.18% of haemolymph samples. This is the first report of *N. ceranae* DNA in honey bee haemolymph and in *V. destructor* mites. The finding of DNA of *N. ceranae* in *V. destructor* could be interpreted as the result of mite feeding on *N. ceranae* infected bee haemolymph. However, for a full confirmation of the vector role of *V. destructor* in spreading of nosemosis, further microscopy investigations are required for the detection of spores in both investigated matrices (haemolymph and *V. destructor* internal tissues).

Key words: *Nosema ceranae*, *Varroa destructor*, haemolymph, *Apis mellifera*, PCR.

INTRODUCTION

Microsporidian parasites *Nosema apis* and *N. ceranae* are among the most widespread honey bee parasites [1,2]. The disease caused by these parasites is not only regional, but also a global problem due to its high prevalence [3-5]. Nosemosis causes pathological

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changes in honey bees, both at individual and colony level, influencing also the productivity of the colonies [4].

Both microsporidian species that infect honey bees (*N. apis* and *N. ceranae*) reproduce only in the midgut epithelial cells of the host [1,6]. However, spores of either *Nosema* spp. were detected in other bee organs such as ovaria, salivary, mandibular and hypopharyngeal glands [7-9], honey [3,12], corbicular pollen [13], bees' wax [14], royal jelly [15], beekeeping material [16], as well as in regurgitated pellets of *Merops apiaster* [17,18], and DNA in salivary, mandibular and hypopharyngeal glands [7-9], Malpighian tubules, fat body [10] and brain [11].

Having in mind that nosemosis in honey bees, especially caused by *N. ceranae,* can cause great losses in beekeeping [19-21], and that microsporidian spores can be found in numerous media in the bee environment, it is of great importance to investigate all possible nosemosis transmission routes. The reports of *Nosema* spp. spores in honey bee haemolymph and different glands [7-9,22] and findings of parasite DNA in various honey bee tissues [9-11] indicate that transport via haemolymph is the only possibe way of transmission of *Nosema* spp. spores from the midgut to anatomically distant tissues and organs of the host bee.

During the last couple of decades, the mite *Varroa destructor* [23] has been considered as one of the main causes of honey bee colony losses. This ectoparasite has spread on almost all parts of the globe, including Serbia [24], so nowadays it is difficult to find a honey bee colony without *V. destructor* [25]. *V. destructor* mites are in very close contact with honey bees from their earliest developmental stages throughout the whole life cycle, and they feed exclusively on honey bee haemolymph. *V. destructor* could transfer honey bee viruses through haemolymph [26], but it is not clear can it serve as a vector of other honey bee endoparasites. For that reason, *V. destructor* mites and bee haemolymph were examined for the presence of *Nosema* spp. using polymerase chain reaction (PCR), the method generally regarded the most sensitive and reliable in detecting small amounts of parasite DNA [27,28]

MATERIAL AND METHODS

Samples

Honey bee (*Apis mellifera*) samples were taken from 44 colonies, all from the same apiary located in Belgrade, Serbia. All colonies were moderately infested with *V. destructor* mites, although they were regularly treated with varroacides. No treatment against *Nosema* spp. was conducted and colonies were confirmed to be *Nosema* spp.-infected. At least 50 adult bees from each colony were randomly collected from honey combs and put into a glass jar with a lid having air openings. Ten bees from each sample were separated for the purpose of haemolymph collection. The rest of the bees were left in a glass jar and shaken vigorously until the live mites were dispatched

at the walls of the jar. At least five live *V. destructor* individuals per hive were collected and processed immediately (without storage).

Haemolymph collection

During the procedure of haemolymph collection, all precautions were undertaken in order to avoid its external contamination. The surface of each individual bee was sterilized as it was described by Huang and Sloter [6]. Briefly, each bee was washed twice in cold 0.09% sodium hypochlorite with 0.2% Tween20, and then rinsed twice in sterile water with 0.2% Tween20. The bees were dried on a clean paper towel and held in a refrigerator for several minutes until they were motionless. After chilling, the bees were allowed to recover enough to pump their abdomens to make haemolymph collection easier. Each bee was immobilized with pins crossed between the abdomen and thorax on a Petri dish full of solid wax. To collect the clear and slightly yellow haemolymph, a small incision (with a sterile needle) was made between the second and third abdominal terga from the thorax, and 2–3 μL of haemolymph per bee was taken using a 10 μL pipette by inserting its tip into the bee at the site of incision. Any sample that seemed unclear (contaminated with gut content) was immediately discarded. A total of 10 μL per hive was obtained from 4-5 bees. Fresh haemolymph was used immediately for DNA extraction.

DNA isolation

Individual mites were sterilized in cold 0.09% sodium hypochlorite with 0.2% Tween20, and then rinsed twice in sterile water with 0.2% Tween20. A total of five mites from each hive were crushed with a sterile pestle in a 1.5 ml tube with 1 mL of distilled water. Afterwards, the suspension was centrifuged (5 min at 16 000 x g), and the supernatant was removed. The precipitate was frozen in liquid nitrogen, crushed with a sterile pestle and used for DNA extraction using "DNeasy Plant Mini Extraction Kit" (Qiagen, Cat. No. 69104). The same chemicals were used for DNA isolation from the haemolymph samples. Isolates were kept at -20°C until PCR analysis.

Nosema spp. detection and species identification

For *Nosema* spp. detection PCR was applied using nos-16S-fw/rv primers [29]. Samples that were confirmed to be *Nosema* spp.-positive were subjected to duplex PCR with species-specific primers (321APIS-FOR/REV for the detection of *N. apis* and 218MITOC-FOR/REV for the detection of *N. ceranae*) designed by Martín-Hernández et al. [19] and PCRs were performed the same way as previously described in detail [29]. As positive controls, reference DNA extracts (from bee macerates) of *N. apis* and *N. ceranae*, while double-distilled water (ddH_2O) was the template for the negative control.

All PCR amplifications were performed in Mastercycler Personal (Eppendorf) and MultiGene Gradient (Labnet International Inc.). PCR products were mixed with

Midori Green Direct (Nippon Genetics, Cat. No. MG06), analysed by electrophoresis on 2% agarose gel (1x TBE) and visualized under UV light. The commercial 100 bp DNA ladder (Nippon Genetics, Cat. No. MWD100) was used for the determination of the PCR fragments size.

RESULTS

The presence of *Nosema* spp. (Table 1) was confirmed in 27 *V. destructor* samples (61.36%) and in 30 haemolymph samples (68.18%). The method used for the detection of *Nosema* spp. DNA in honey bee mite is based on usage of nos-16S-fw/rv primers capable for simultaneous detection of *N. apis* and *N. ceranae* (Figure 1). To determine *Nosema* species, all samples revealed to be *Nosema* spp.-positive (27 samples of *V. destructor* and 30 haemolymph samples) were analysed with duplex PCR which revealed only *N. ceranae* (Figure 2). No infection with *N. apis* was diagnosed, and no mixed infection was detected (Figure 2). As expected, the length of PCR products in the reference samples (bee macerates) corresponded to *N. apis* and *N. ceranae* positive controls, whilst no signal was detected in the negative control.

Table 1. *Nosema* DNA presence in *Varroa* mites and honey bee haemolymph

	Number (%) of Nosema spp. positive Varroa samples			**Number (%) of Nosema spp. positive haemolymph samples**		
PCR detection of *Nosema* spp.	27 (61.36)			30 (68.18)		
Species identification	*N. apis*	*N. ceranae*	Mixed infection	*N. apis*	*N. ceranae*	Mixed infection
	0 (0)	27 (100)	0 (0)	0 (0)	30 (100)	0 (0)

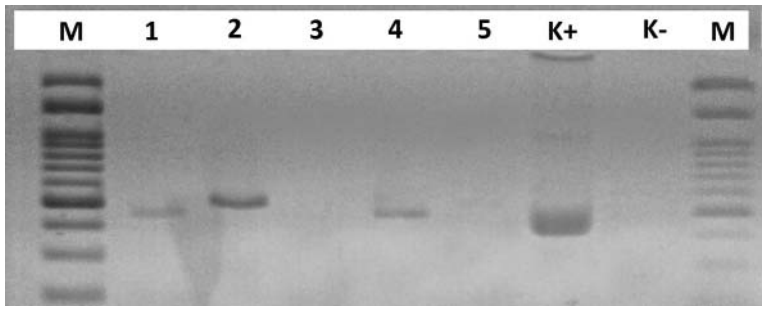

Figure 1. Detection of *Nosema* spp. PCR products generated with primers nos-16S-fw/rv (488 bp).
Lanes 1 and 2, haemolymph samples infected with *Nosema* spp. Lane 4, *V. destructor* sample infected with *Nosema* spp., Lanes 3 and 5, *Nosema* spp.-free haemolymph and *V. destructor* samples, respectively, Lane K+, positive *Nosema* spp. control., Lane K-, negative control, Lanes M, 100 bp DNA ladder.

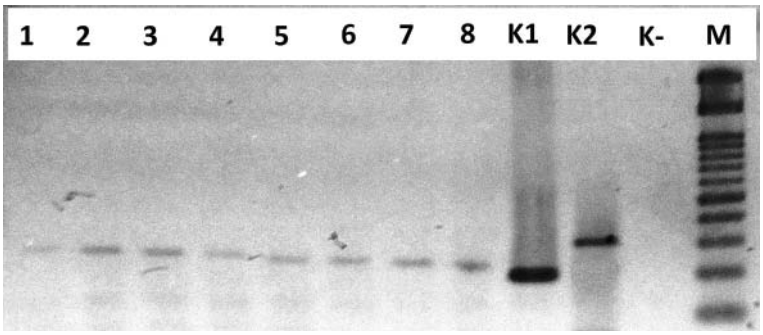

Figure 2. Detection of the 16S rRNA duplex PCR products that allow *N. apis*/*N. ceranae* differentiation.
Lanes 1-4, haemolymph samples infected with *N. ceranae* (218 bp), Lanes 5-8, *V. destructor* samples infected with *N. ceranae* (218 bp), Lane K1, positive *N. ceranae* control, Lane K2, positive *N. apis* control (312 bp), Lane K-, negative control, Lane M, 100 bp DNA ladder.

DISCUSSION

Massive losses of honey bees documented during the last decade all over the world have drawn attention and encouraged a large number of studies with an aim to find the causes of these losses and to solve the problem. However, a single factor which could be the only and secure cause of so-called „unexplained" honey bee losses, has not been detected so far [30]. Therefore, the current opinion is that multiple biological and abiotic factors, alone or in combination, can lead to dramatic losses of honey bee colonies [31]. The honey bee pathogens have an important role in this phenomenon [30]. Pesticides and climate parameters, as well as their interactions and possible synergism [31] are also taken into account. The ectoparasite *V. destructor* is the most frequently mentioned as the main cause of winter losses of honey bees in cold climate conditions [32,33], while harmful effects of microsporidian *N. ceranae* are more important in the Mediterranean climate [4]. Except as single factors, *V. destructor* and *N. ceranae* may contribute to colony collapse as co-factors acting synergistically with other factors, mostly viruses [33,34] or pesticides [35,36]. After all, there is still no acceptable treatment or strategy for the control of *Varroa* spp. and *Nosema* spp. parasites, mainly because of drug side effects [37,38].

The search for the presence of *Nosema* spp. DNA in honey bee mite *V. destructor* was based on previous findings of *Nosema* spp. spores and DNA in host tissues [9-11] that are anatomically distant from the bee midgut where *Nosema* spp. persist. As hemolymph represents a medium which connects the midgut with other bee tissues, we presumed a possible distribution of microsporidian parasites by haemolymph. For that reason, the finding of *Nosema* spp. DNA in the haemolymph was expected. The finding of *N. ceranae* DNA in *V. destructor* mites was expected as adult *V. destructor* females feed on honey bee haemolymph.

This paper confirms the presence of *N. ceranae* DNA in bee haemolymph for the first time, in contrast to recents attempts of Huang and Solter [6] who failed to detect DNA of either *N. apis* or *N. ceranae* in the same medium. Moreover, in this study *N. ceranae* DNA was found in *V. destructor* mites for the first time. This can be compared with findings of other *Nosema* species in many other mite species, taxonomically realted to *V. destructor* [39].

It is well-known that *V. destructor* mite is very mobile and it can move from one to the other honey bee individual, capable to serve as a vector of a large number of honey bee viruses [26,30]. That is why *V. destructor* is considered the most significant honey bee pathogen [25]. Our finding of *N. ceranae* DNA in *V. destructor* opens the question of its vector role in nosemosis spreading. However, without microscopic detection of spores in both investigated matrices (haemolymph and mite internal tissues) it is impossible to assert that *V. destructor* serves as biological vector of nosemosis.

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DNK *Nosema ceranae* U HEMOLIMFI PČELA I PČELINJEM KRPELJU *Varroa destructor*

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Pčelinji krpelj *Varroa destructor* i mikrosporidija *Nosema ceranae* su najznačajniji faktori rizika po zdravlje pčela i pčelarstvo. Dugo se smatralo da *N. apis* i *N. ceranae* inficiraju isključivo ćelije crevnog epitela pčela, međutim neki nalazi ukazuju na prisustvo ovih mikrosporidija i u drugim tkivima. Postoje indikacije da se ovi patogeni mogu naći i u hemolimfi pčela, kao medijumu za njihovu distribuciju do anatomski udaljenih tkiva.

S obzirom na to da se *V. destructor* kao ektoparazit hrani hemolimfom pčela, cilj ovog rada bio je ispitivanje prisustva DNK mikrosporidija roda *Nosema* u hemolimfi pčela i pčelinjem krpelju *V. destructor*.

Istraživanje je obavljeno na uzorcima pčelinje hemolimfe i pčelinjim krpeljima *V. destructor* iz 44 društva *Apis mellifera*. Iz svake košnice je uzorkovano pet krpelja i 10 μL hemolimfe (sakupljene iz 4-5 pčela) koji su korišćeni kao uzorci za izolaciju DNK i ispitivanje prisustva *Nosema* spp. putem PCR metode.

Prisustvo DNK *N. ceranae* je potvrđeno u 61,36% analiziranih krpelja *V. destructor* i 68,18% uzoraka hemolimfe pčela, što predstavlja prvu detekciju prisustva DNK *N. ceranae* u hemolimfi pčela i krpeljima *V. destructor*. Nalaz DNK *N. ceranae* u *V. destructor* može se tumačiti kao rezultat ishrane *V. destructor* hemolimfom pčela koja je inficirana sa *N. ceranae*. Međutim, da bi se potvrdila vektorska uloga *V. destructor* u širenju nozemoze, potrebna su dalja mikroskopska istraživanja radi detekcije spora u oba ispitivana medijuma (hemolimfi i unutrašnjim tkivima *V. destructor* krpelja).