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RESEARCH PAPER

Detection of *Varroa destructor* Mite and *Nosema* spp. in Bee Samples From Bulgaria

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Abstract

This study is focused on the investigation of honey bee samples for the presence of the two most common and widely distributed honey bee parasites. In a two-year period during 2020-2021, 185 bee samples were tested. All samples were examined by morphological and light microscopic methods. The obtained results showed that 32.43% of bee samples were infested with *Varroa destructor.* The degree of infection in the bees ranged from 0.5% to 60%. Spores of *Nosema* spp. were established in 25.40% of samples with a degree of invasion in the range from $3x10^5$ to $26x10^6$ per bee. Mixed infections of both parasites were observed in 32.43% of the samples. Negative samples were with the lowest value of 9.74%.

Introduction

The managed honey bee (*A. mellifera*) considerably contributes not only to crop pollination, but and many wild flowering plants (Botías et al., 2013; Hristov et al., 2020a). Maintaining the health of honey bee colonies is of great importance for pollination and agriculture sustainability. Crop yields are influenced by the density and quality of honey bee colonies placed in fields, groves, and orchards (Calderone, 2012; Chatterjee, 2021; Lowe et al., 2021).

Beekeeping is facing many challenges. On the one hand the impact of the increasingly unfavorable for the bees as environmental conditions (climate change, lack of feed, insufficient variety of food, environmental pollution, pesticides etc.). On the other hand, many honey bee pathogens and pests have a significant impact on honey bee health and survival (Neov et al., 2019; Hristov et al., 2020b). The ectoparasitic mite *Varroa destructor* contributes to the higher levels of bee losses around the world (Ramsey et al., 2019; Chen et al., 2021). Climate change induces longer periods of brood rearing in honey bee colonies and foraging because of longer warm seasons. A longer brood period means more *Varroa* reproduction cycles and may lead to an increase in mite populations (Le Conte et al., 2010; Beaurepaire et al., 2017). Also, the *Varroa* mite potential to act as vectors of honey bee associated viruses (Barroso-Arévalo et al., 2019). Several bee viruses, including Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus (CBPV), Deformed Wing Virus (DWV), Kashmir Bee Virus (KBV), Sacbrood Virus (SBV) and Israeli Acute Paralysis Virus (IAPV), have been documented to be aggravated and transmitted by *Varroa* mites (Annoscia et al., 2019; Erban et al., 2019; Roberts et al., 2020). Nosemosis caused by the microsporidia *Nosema apis* and *Nosema ceranae* are among the most common pathogens affecting adult honeybees. Especially, *Nosema ceranae* infection is associated with annually honey bee losses worldwide (Rubanov et al., 2019; Shumkova et al., 2021). One of the main reasons for the high losses of bee colonies are varroosis and nosemosis, the most prevalent and damaging infection diseases of honeybees, which cause large losses in beekeeping. For this reason, this study aimed to evaluate the presence of *Varroa destructor* and *Nosema* spp. in honey bee samples in different regions of Bulgaria.

Materials and Methods

In a two-year period (2020-2021), in the private laboratory "Primavet - Sofia Ltd." and in the Department of Experimental Parasitology, IEMPAM-BAS were tested 185 bee samples from apiaries, located in different regions of the country. The private laboratory "Primavet - Sofia Ltd." surveyed 94 samples for diagnosis, mainly from bee colonies with some pathological symptoms such as abnormal behavior, depopulation of beehives, weakness and high losses of colonies. In the Department of experimental Parasitology were tested microscopically 91 bee samples for monitoring of *Varroa destructor* and *Nosema* spp. infestation rate. Bee samples were taken personally by beekeepers or a veterinarian. In the case of dead bee colonies, the method for diagnosing varroosis was by examining the debris generated by the bees themselves, where fallen/dead mites can be found. For investigation of nosemosis samples of older worker honey bees were taken from the hive entrance or from external frames if the weather does not permit flight conditions. For investigations of varroosis from colonies with some pathological problems, beekeepers selected any uncapped brood frame and check that the queen is not present and take a sample of 300 bees using a jar (with a mark at the level of 100 mL of water, which is the volume occupied by 300 bees) by sliding it up and down so that the bees tumble in. The beekeepers then store the samples until they are sent by cooling at 4°C for 15 minutes or by freezing to –18°C for 5 minutes.

Identification of *Varroa destructor*, and *Nosema* spp. were performed according to instructions described by OIE Terrestrial Manual (2021) and OIE Terrestrial Manual (2013), respectively. Diagnostic methods used to detect *Varroa destructor* morphological identification by alcohol wash method (Oliver, 2020) and to proof spores of *Nosema* spp., included light microscopic (×400 magnification) according to Fries et al. (2013).

Investigation for *Varroa destructor* Mite

The dead bees from the bee samples were flooded with industrial alcohol and stirred continuously for around 5-10 minutes. Then the contents were placed of the sieve on a bright plate, where the mites can be easily identified and counted. The percentage of a mite infestation level was calculated by the following formula:

% *V. destructor* = (Number of foretic mites/number of adult bees) × 100

The results can be expressed as a percentage of infestation, dividing the number of mites dislodged by the number of bees in the sample and then multiplying by 100.

Investigation for Spores of *Nosema* spp.

The abdomens of sixty bees of each sample were obtained in 60 mL of distilled water. Smears of the suspension were made on a glass slide. They were air-dried, ethanol-fixed and stained with Giemsa's stain (10% in 0.02 M phosphate buffer) for 45 minutes. *Nosema* spp. spores had a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. To quantify the average infection level spores were counted and were calculated per bee as the abdomens of 60 individuals are macerated in 5 mL of water using a mortar and pestle and 50 mL of water was added for a total volume of 1 mL per bee (5 mL is added later). When tissue pieces have become quite fine, the suspension was filtered through two layers of muslin (thin loosely woven cotton fabric) in a funnel leading to a graduated centrifuge tube. A second 5 mL of water was used to rinse the pestle, swirl around the inside of the mortar and pour through the subsample in the funnel. When the suspension appeared to be homogenous after shaking, a sample was taken to fill the calibrated volume under the cover-slip of a haemocytometer (blood cell counting chamber). After a few minutes, the spores will have settled to the bottom of the chamber. *Nosema* spp. spores appear transparent but with a very distinct dark edge and are 5–7 µm long and 3–4 µm wide. They were counted using a magnification of ×400. The number of spores in each square was counted. The whole grid consists of 3 × 3 large squares, separated by triple lines. Each large square is further subdivided into 16 smaller squares subdivided by double lines, in total 144 squares. The spores are counted in the smaller squares with the area of 1/25 mm^2. When the counting is completed, the number of spores per bee in the sample can be calculated according to the formula:

$$\mathbf{Z=\alpha/\beta\times\delta\times 250\ 000}$$

Where

Z = spore numbers per bee

α = total number of spores counted

β = number of squares counted

δ = dilution factor

The number 250 000 was used because the volume in each counted square was 1/250 000 mL and the equation uses the average number of spores per counted square.

Results

Results from the private laboratory "Primavet - Sofia Ltd." have shown in Fig.1. It can be seen that 32.98% of bee samples were infested with *Varroa destructor.* The degree of invasion in bees was in the range of 0.5% to 60%. Spores of *Nosema* spp. were established in 26.60% of samples with a degree of infection ranged from $3x10^5$ to $26x10^6$ per bee. Mixed infections of both pathogens were observed in 28.72% of the analyzed samples, while the negative samples were 11.70%.

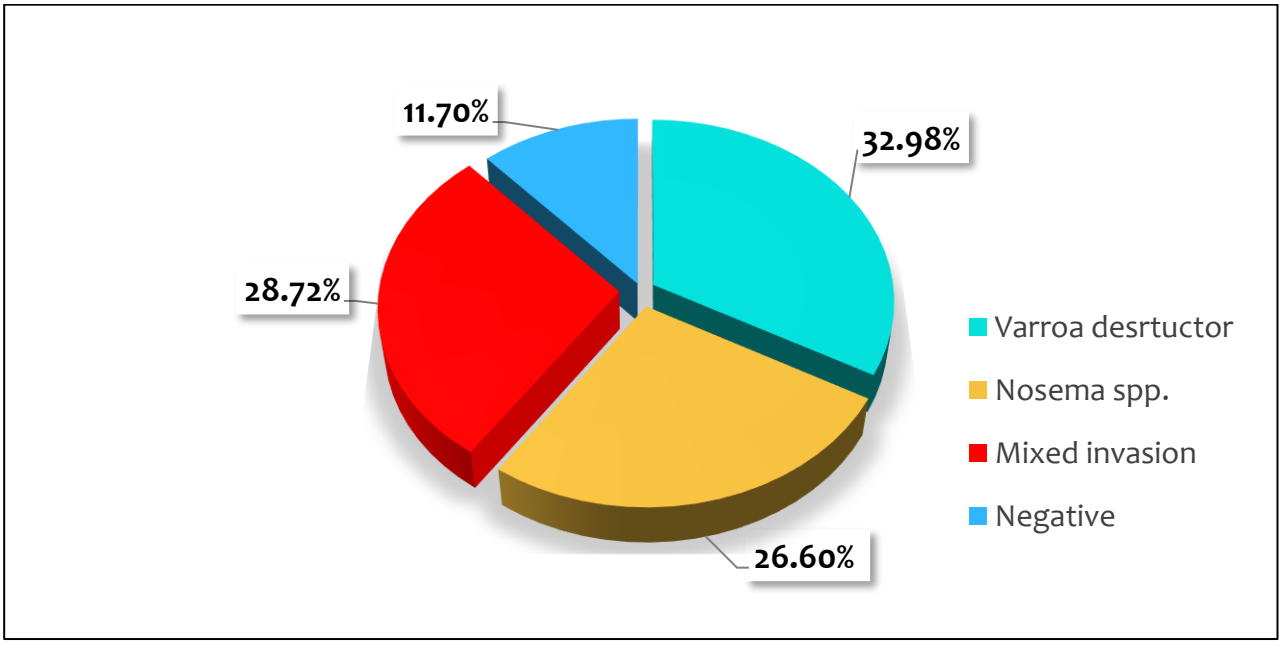

Figure 1. The results of investigated bee samples from the private laboratory "Primavet"

Results from ninety-one investigated samples in the experimental laboratory (IEMPAM – BAS) (Fig. 2) showed the highest percentage of mixed infections (36.26%), samples infested with *V. destructor* were 31.87%, and spores of *Nosema* spp. were identified in 24.18% of honey bee samples. Negative samples were only 7.69%.

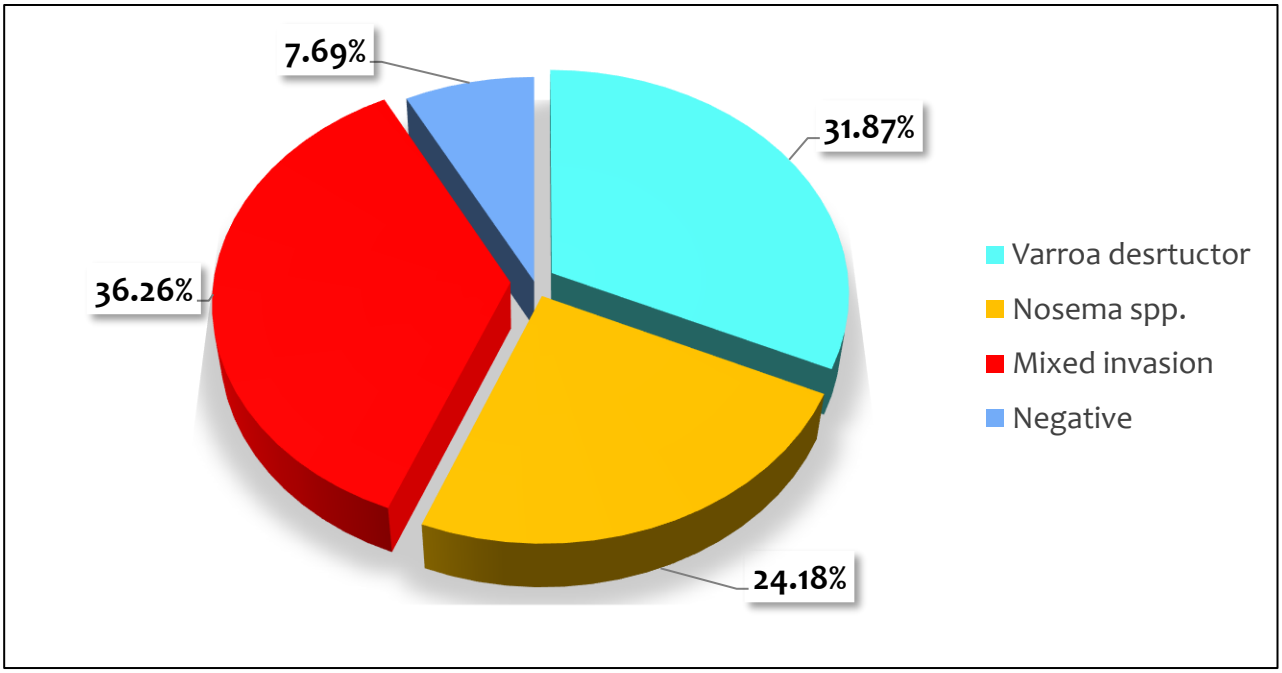

Figure 2. The results of investigated bee samples from the experimental laboratory

Figure 3 presents the obtained results from tested honey bee samples from different regions in the country.

The majority of samples were obtained from the Pleven, Sofia, Sofia-province, Pazardzhik, Ruse, Razgrad, Veliko Tarnovo, Stara Zagora, Blagoevgrad and Burgas regions. The large number of samples obtained from a given area is not always in direct correlation with high percentage of positive for *V. destructor* or *Nosema* spp. samples from the same area. The highest percentage of positive samples for *V. destructor* was found in Targovishte (100%), Blagoevgrad (71.4%), Stara Zagora (71.4%), Sofia-city (55.6%), and the lowest infestation rate was observed in Sofia province (12.5%), Pazardzhik (14.3%) and Ruse (20%). In some regions there was no detected *Varroa* positive samples (Gabrovo and Lovech) (Fig. 3). The highest percentage of positive samples for *Nosema* spp. was found in Lovech (100%), follow by Gabrovo (75.0%), Veliko Tarnovo (62.5%), Razgrad (50.0%) and Ruse (40.0%). The *Nosema* spp. infection did not observe in Vidin and Targovishte. The mixed invasion was observed most often in samples sent by beekeepers from Sliven, Pazardzhik, Sofia-region and Ruse (Fig. 3).

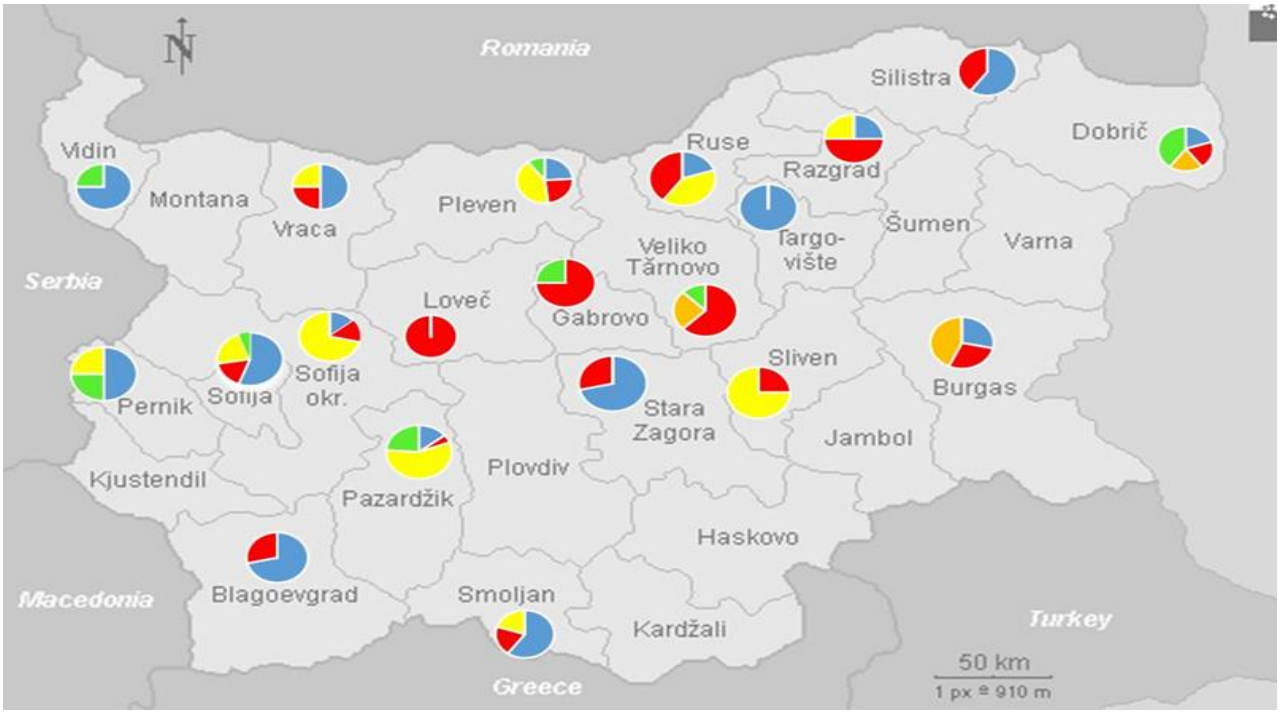

Figure 3. Distribution (%) of *Varroa destructor* mite and *Nosema* spp. in honey bee samples from different locations in Bulgaria.

*****Blue color - honey bee samples positive for *V. destructor*; red color- honey bee samples positive for *Nosema* spp.; yellow color - mixed invasion, green color - negative samples.

Discussion

The ectoparasite mite *Varroa destructor* has caused severe damage to populations of the European honeybee, *Apis mellifera* worldwide in recent years. In our studies, we found a very high percentage of bee colonies infested with both *Varroa destructor* mite and *Nosema* spp. The data are not representative for the whole country due to the different number of samples obtained from the various areas. The highest rate of *V. destructor* infestation was observed in plain regions of the country (Targovishte, Stara Zagora and Vidin) (Fig. 3). One possible explanation is associated with significantly longer honey bee brood rearing in the plain regions compared to mountainous parts – from early spring (February) to late autumn (October). This gives an opportunity to the prolonged influence of the mite on honey bee colonies.

The obtained results showed that *Nosema* spp. infection also prevailed in the plain regions in the country (Gabrovo, Lovech and Razgrad). In the plain regions compared to the mountainous, the transport of infected honey bees and/or by the increased mobility of people, goods and livestock are observed much more often. Thus, it is a possible explanation of higher *Nosema* spp. infestation rate in the plain regions of the country. In our preliminary studies, we found that most of the tested samples of dead bees were infested with *Nosema* spp. (Salkova et al., 2015; Salkova et al., 2016a; Salkova et al., 2016b). The results of our previous research showed the predominant part of the research the samples showed the presence of *V. destructor* and *N. ceranae* in Central and Northeastern Bulgaria. In addition, in the regions observed in 2020, 37% of the samples show mixed infestation of varroosis and nosemosis, 33.3% - only varroosis and 14.8% - only

nosemosis (Salkova et al., 2022). According to the Bulgarian Food Safety Agency (BFSA), the results of an epizootological survey in different areas of the country in 2020 showed that bee colonies are most often affected by varroosis and nosemosis, while in the previous year varroosis predominated as a cause of mortality in honey bee colonies.

The reason of this result might be connected with beekeeper's under estimate *Varroa* infestation levels in their apiaries, and often beekeepers' management of infestations is failing. In order to control the level of *Varroa destructor* infestation, beekeepers must start control treatment when monitoring and controlling varroosis before the number of mites is high enough to cause significant harmful effects on the bee colony. As is the case for mite population dynamics, damage thresholds are highly variable and depend on the interaction between genotype and environment together with beekeeping management practices and the time of year, resulting in substantial differences between regions (Rosenkranz et al., 2010). In recent years, the presence of mite resistance against certain groups of acaricides is becoming all too common in manly areas of the world. To effectively control of varroosis, beekeepers should be interested in new veterinary medicinal products (VMPs) and check for resistance to the product used in their apiary. A reliable and harmonized diagnosis is crucial to ensure the quality of surveillance and research results.

Conclusion

In conclusion, we can say that our study has shown a prevailing higher percentage of infested with *Varroa destructor* mite bee samples than samples, positive for *Nosema* spp. in our country. We should also note the relatively high percentage of samples with mixed infection. A small percentage of the tested samples were negative.

Ethical Statement

Not applicable.

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No funding was received to assist with the preparation of this manuscript.

Author Contributions

Prof. Kalinka Gurgulova tested the bee samples sent to the private laboratory of "Primavet".

Chief Assist. Delka Salkova tested the bee samples in the laboratory of Experimental

Parasitology for monitoring of bee parasites and has prepared the manuscript.

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