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# NOSEMOSIS TYPE C OF BEES CAUSED BY MICROSPORIDIA *Nosema* (*Vairimorpha*) *ceranae*: CURRENT VIEWS, PATHOGENESIS, PREVENTION, DIAGNOSIS AND TREATMENT

(review)

**S.A. TIMOFEEV ✉, A.N. IGNATIEVA, V.V. DOLGIKH**

*All-Russian Research Institute of Plant Protection*, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail ts-bio@ya.ru (✉ corresponding author), edino4estvo@mail.ru, dol1slav@yahoo.com
ORCID:
Timofeev S.A. orcid.org/0000-0001-6664-3971
Dolgikh V.V. orcid.org/0000-0002-2362-2633
Ignatieva A.N. orcid.org/0000-0001-7288-4077
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## Abstract

Nosemosis type C is a parasitic disease of honey bees caused by the obligate intracellular parasite microsporidia *Nosema (Vairimorpha) cerana*. This disease is widespread worldwide and can lead to a decrease in honey production, a sharp reduction in the population of adults in bee families and their final death (M. Higes et al., 2007; P.J. Marín-García et al., 2022). The purpose of this review is to present up-to-date data on this disease and its causative agent, as well as on modern methods of diagnosis, prevention and treatment in beekeeping. The parasite is mainly transmitted between bees by the fecal-oral route and infects the cells of the middle intestine of insects (R. Galajda et al., 2021).. Vertical transmission of the parasite is also possible, as *N. ceranae* spores have been found in ovarian cells of infected queens (C. Alaux et al., 2011). The pathogenesis of *N. ceranae* is associated with the destruction of infected cells, the restructuring of the host's metabolic processes to meet the needs of the parasite, the shortage of spare resources and vital metabolites in sick bees. hormonal imbalance; negative consequences of part of the immune responses to the pathogen invasion, such as oxidative stress (L. Paris et al., 2017). Ability of *N. ceranae* specifically inhibits such protective reactions of bees as activation of apoptosis of infected cells and production of antimicrobial peptides can enhance the pathogenic nature of nosemosis type C (K. Antunez et al., 2009; C. Kurze et al., 2015). The method of diagnosis of infection includes the primary detection of the parasite using light microscopy, including with the use of various dyes (N.J. Ryan et al., 1993), and further determination of the species of microsporidia using molecular methods such as standard polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP). The most effective drug for the treatment of nosemosis of bees for a long time remained the antibiotic fumagillin, despite the fact that *N. ceranae* can acquire resistance to this drug (W.-F. Huang et al., 2013; I. Tlak Gajger et al., 2018). However, the discovery of residues of this drug in honey produced by bees after treatment and its toxicity to humans led to the prohibition of this drug in a number of countries and the cessation of its production in 2018 (I. Tlak Gajger et al., 2018). In this regard, many studies have been conducted in recent years aimed at finding new ways to treat nosemosis. For example, extracts from various fungi and plants, probiotics such as eugenol, chitosan, naringenin, proteksin, proteasome function inhibitors ixazomib, and ixazomib citrate are considered as agents for the treatment of this disease (V. Chaimanee et al., 2021; S.S. Klassen et al., 2021; E.M. Huntsman et al., 2021). Despite the fact that many of the tested methods have shown encouraging results, a safe analogue of fumagillin, similar to it in terms of the effectiveness of the fight against nosemosis, has not yet been found. The article also provides recommendations for the care of beehives for the prevention of nosemosis type C in beekeeping.

Keywords: *Nosema ceranae*, *Vairimorpha ceranae*, *Apis mellifera*, nosemosis, microsporidia, bee diseases

Pollination of plants by insects is of key importance for agriculture and the Earth biosphere as a whole. Bees are one of the main pollinators [1], and the proportion of crops pollinated by them can be as high as 53% in some countries [2]. In recent years, the European continent has seen a downward trend in pollinator populations, in particular the honey bee *Apis mellifera* Linnaeus, 1758 [3-6]. The reason for this trend may be a number of abiotic and biotic factors, e.g., the intensification of agriculture and the widespread use of chemical insecticides, the spread of transgenic plants and their pollen resistant to phytophagous insects, global climate change, etc. Diseases caused by various pathogens and parasites have been cited as a possible reason for the decline in honey bee populations [6, 7].

*Nosema ceranae* is an obligate intracellular parasite of bees, belonging to microsporidians (a group of unicellular organisms related to fungi). The species was first described in 1996 in the Chinese wax bee *Apis cerana* [8] and is now considered to be infectious in the taxa of stingless bees (Meliponini), true wasps (Vespidae), and some species of bumblebees and bees, including all subspecies of the honey bee *A. mellifera* [9-12]. A 2020 phylogenetic revision of the genera *Nosema* and *Vairimorpha* showed that *N. ceranae* is correctly classified in the second genus [13]. However, since the vast majority of modern works continue to use the traditional name of the parasite *Nosema ceranae*, we will also stick to this name for the purposes of this review.

*N. ceranae* is widespread in beekeeping in most countries. It is considered the dominant microsporidia species infecting honey bees and has displaced N. apis, the natural pathogen of *A. mellifera* [14-16]. The disease caused by this parasite, Asian nosematosis, or type C nosemosis, has been associated with sharp declines in the adult population of bee colonies, decreased honey production, and even colony collapse [12, 17].

The purpose of this review is to present current data on Asian nosematosis and its causative agent, as well as modern methods of its diagnosis, treatment and prevention in beekeeping.

Biology and pathogenesis of *Nosema ceranae*. The infectious stage of the life cycle of *N. ceranae*, capable of existing in the external environment, like all microsporidia, is spores, the size of which is about 4.7×2.7 μm [8]. The spores enter the bee's body with food, and probably through contact with other infected bees, for example during grooming [18]. During this process, the bees groom each other, which helps them get rid of other parasites such as *Varroa destructor*, but grooming can also lead to the spread of nosematosis. In the midgut of the insect, protected only by the peritrophic membrane, the embryo (sporoplasm) of the parasite is introduced into the epithelial cell using a complex spore extrusion apparatus. Inside the host cell, the sporoplasm transforms into a meront, and after several cycles of division, the process of formation of new spores begins, which are released from the infected cells into the intestinal lumen when enterocytes are destroyed and serve as a source of infection of other cells of the same insect or other individuals. The discovery of empty shells of *N. ceranae* spores that do not contain the parasite embryo in the intestinal epithelial cells of bees may also indicate that extrusion of spores and infection of neighboring insect intestinal cells takes place inside the infected cell [19, 20].

Vertical transmission of the parasite is also possible, as *N. ceranae* spores have been found in ovarian cells of infected queens [21, 22]. Infection of these cells usually occurs through contact with infected worker bees [23], and drones are infected in the same way [24]. It is believed that in natural and commercial hives only adult bees are infected, however, in laboratory conditions, the development of nosematosis has been shown in prepupae infected with *N. ceranae* at the 3-day larval stage [25].

Although *N. ceranae*, in addition to the intestines, has been found in various organs of worker bees [26, 27], their infection has never been proven histologically. The authors [28] of a recent detailed study of *N. ceranae* tropism in the honey bee suggest that the results described above were due to contamination of tissue samples with parasite spores due to imperfect dissection techniques or destruction of the insect intestine at a late stage of infection. That is, most likely, the intestine serves as the only organ in which *N. ceranae* develops [28]. In this case, the development of the parasite occurs only in enterocytes, but not in the stem cells necessary for the renewal of the intestinal epithelium [29].

Both under natural conditions and during artificial infection with *N. ceranae*, a pathology with similar symptoms occurs in the intestine of the infected insect [30]. Infected epithelial cells show signs of degradation, e.g., the appearance of vacuoles in the cytoplasm, disruption of the integrity of cell membranes, condensation and a decrease in the size of the cell nucleus, usually accompanied by hyperchromatosis (excessive increase in chromatin content) and pyknosis (shrinkage of the cell nucleus during chromatin condensation). The peritrophic membrane disappears completely or becomes significantly fragmented. In the underlying region of the brush border, rupture of the cell plasma membrane is sometimes observed. In the most infected epithelial cells, the nucleus is displaced apically. In some host cells, immature and mature stages of *N. ceranae* can be found in invaginations of the nuclear envelope. Lytic processes are actively occurring in infected cells, as evidenced by numerous vacuoles and aggregates of ribosomes and lysosomes, as well as the loss of glycogen particles [19, 30]. Loss of glycogen and loose aggregation of ribosomes are possible consequences of mitochondrial damage [31]. The utilization of glycogen stores may indicate that the infected cell has switched from a more efficient mode of energy production (e.g., oxidative phosphorylation) to a less efficient anoxybiont glycolysis, perhaps to compensate for the depletion of ATP that is taken up by the parasite from infected cells [32]. ATP deficiency leads to disruption of ion transport across the membrane of the infected cell, which leads to the accumulation of excess sodium in the cell, detachment of ribosomes from the rough endoplasmic reticulum and, ultimately, to necrosis [14, 31].

Impact on the host cell metabolism and its specific reconfiguration to increase the availability of nutrients and energy resources for the parasite is a characteristic feature of microsporidia parasitism [32]. When intestinal cells are infected with *N. ceranae*, *A. mellifera* exhibits upregulation of the alpha-glucosidase gene and three genes involved in trehalose transport, as well as downregulation of genes encoding trehalase and glucose-methanol-choline oxidoreductase [33, 34]. These metabolic changes result in increased availability of trehalose to the parasite, which is considered the main source of glucose for microsporidians [32].

*N. ceranae* also specifically affects host defense responses at the cellular level. Infected intestinal cells may undergo apoptosis before the parasite has time to complete its full developmental cycle. *N. ceranae* appears to be able to block this process, as evidenced by increased transcription of various apoptosis inhibitors in infected cells [33-35]. This assumption is supported by the fact that in a nosematosis-resistant line of bees, no inhibition of apoptosis was observed during experimental infection [36]. Another cellular mechanism of protection against microsporidia infection is oxidative stress, i.e., the production of reactive oxygen species by infected cells that can destroy intracellular parasites. This mechanism appears to be ineffective against N. ceranae infection because the parasite thrives, despite observed oxidative stress and damage to intestinal cells, by producing catalase, glutathione peroxidase, and glutathione S -transferase [4, 37]. However, it is likely that *N. ceranae* still affects infected bee cells and suppresses oxidative stress responses, since when the latter was artificially induced by pesticides, infection

with *N. ceranae* reduced the amount of reactive oxygen species and reduced damage to intestinal cells by *A. mellifera* [38].

At the organismal level, Asian nosematosis in bees can be asymptomatic or cause significant harm. This largely depends on the external conditions in which bees exist, on fluctuations in temperature and humidity [16, 39]. In the laboratory, infection of bees with a dose of $10^5$ spores per individual led to 100% mortality of insects by day 8 after infection [19]. One of the important consequences of nosematosis in bees is energy stress caused by disruption of the damaged intestine and changes in the carbohydrate metabolism of infected cells. Although infected bees consume more food than uninfected bees, they do not appear to be able to utilize the excess amounts of carbohydrates consumed, most of which are used by the pathogen to complete the life cycle [12, 17, 40]. Energy stress is associated with high mortality of worker bees during nectar collection, which requires significant energy expenditure [40, 41]. Infected bees collect nectar less efficiently than healthy bees and more often do not return to the hive due to impaired orientation in space [42]. Infected worker bees also spend more time outside the hive, engaging in risky behaviors such as robbing [43]. Interestingly, similar effects were not observed when a line of bees resistant to nosematosis was artificially infected [44]. Energy stress due to nosematosis is associated with the degradation of the hypopharyngeal glands in nurse bees, which produce secretions for feeding larvae, and the depletion of the secretion itself [40, 41]. Despite the fact that the death rate of drones is usually lower than that of worker bees, the effects of nosematosis and energy stress are usually more pronounced in them, and their mortality from this infection is higher [45].

A number of other changes in the body of infected bees and the consequences of nosematosis are associated with the immune response to the infection. The oxidative stress described above directly affects the reduced lifespan of infected bees [40]. *N. ceranae* appears to suppress other insect defense responses at the organismal level. Infected bees have reduced expression of many genes associated with the immune response, including those encoding antimicrobial peptides and hormones. Suppression of expression was observed for proteins such as abaecin, defensin, hymenopthecin, glucose dehydrogenase, vitellogenin, serine protease 40, and catalase [34, 46]. In addition, suppression of the expression of components of the immune response activation pathways Toll and Imd and pattern recognition receptors was noted [47]. Similar suppression of the immune response in infected bees was observed not only in laboratory experiments and agricultural hives, but also in natural populations [48]. However, in other studies, on the contrary, upon infection with *N. ceranae*, the expression of various components of the immune response of bees increased, including many of the genes mentioned above [49, 50]. This finding suggests that the immune response may be influenced by many factors, such as infectious load, which varies significantly between studies; duration of tests; biomaterial in which gene expression was studied (whole bees, abdominal cavity, ventricles, etc.); age of bees at infection and during the study. The mechanisms of interaction of *N. ceranae* with the host immune system and their role in pathogenesis require further study.

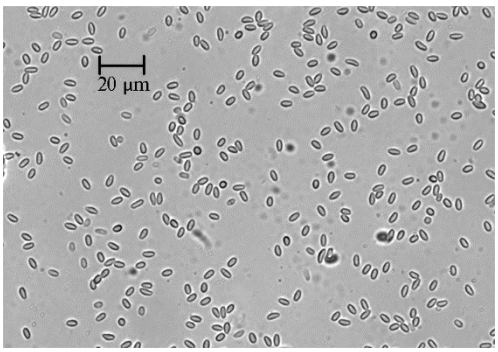
Depending on their age, bees perform different functions in the hive. For example, young bees clean, build, and feed brood within the colony, whereas external tasks are reserved for older bees [51]. *N. ceranae* infection causes hormonal imbalance, leads to behavioral disturbances and accelerates the development of infected bees. The distribution of functions between individuals of different ages is primarily regulated by the ratio of the production of juvenile hormone and vitellogenin in the bee's body. Worker bees infected with *N. ceranae* had increased levels of juvenile hormone, which led to a premature transition from foraging to

foraging outside the hive [51, 52].

Infection with *N. ceranae* may be one of the factors influencing the social demography of a colony. Infected bees die due to destruction of body tissue, are unable to return to the hive due to energy stress, or leave the hive to limit transmission of infection to healthy worker bees. Young bees start searching for nectar earlier due to hormonal imbalance, and the feeding process of larvae and other individuals in the hive is disrupted. The loss of infected worker bees over time can cause accelerated aging and premature foraging in uninfected young foraging insects. Young foragers are less efficient at obtaining food than normal-aged foragers, which becomes a threat to the food security of the colony. The culmination of these effects can ultimately lead to sudden colony death [17, 19, 53].

Detection of *Nosema ceranae* and diagnosis of Asian nosematosis in bees. In a bee colony, clinical and subclinical manifestations of Asian nosematosis are expressed in a longer breeding period in cold months, an increase in the proportion of brood frames relative to the number of nurse bees in warm months, and a decrease in honey production. Infected colonies weaken, the proportion of adult bees decreases, which leads to the death of the colony within 1.5-2 years [12, 54]. None of these manifestations is specific to *N. ceranae* infection and may be caused by other diseases, requiring a differential diagnosis to determine nosematosis [16].

The first step for detecting *N. ceranae* in a hive is microscopic analysis of feces, smears from opened dead bodies, and intestinal homogenates for the presence of spores. Their characteristic ovoid shape (Fig.) makes it easy to identify microsporidia infestations. To facilitate the detection of the parasite, specific dyes, the trichrome and calcofluor white are used in the preparation that bind to the chitin of the shell of microsporidia spores, as well as the nonspecific dye toluidine blue [55, 56].




**Microphotograph of *Nosema ceranae* spores from the intestinal homogenate of an infected bee *Apis mellifera*** (light transmission microscopy, Axio Imager M1, Carl Zeiss, Germany, magnification ×100; photograph taken by A.N. Ignatieva).

Recently, a method for primary microscopic detection of *N. ceranae* in the field without laboratory equipment was proposed. Using a regular smartphone, ultraviolet LEDs, and a set of simple lenses, the authors developed a device weighing 374 g that allows one to effectively detect *N. ceranae* spores in samples when stained with a modern modification of calcofluor white [57]. Spores of *N. ceranae* differ slightly from spores of *N. apis* morphologically (the latter have a more rounded shape), therefore, molecular detection methods are necessary for reliable differentiation of these species [12, 16].

The main method for detecting nosematosis in bees and establishing the specific species of the parasite is standard polymerase chain reaction (PCR) or real-time PCR (qPCR) [58, 59]. To do this, it is necessary to use primers specific for *N. ceranae*, *N. apis*, and also for both species simultaneously. A complete list of primers that are used in the diagnosis of nosematosis can be found in the work of R. Galajda et al. [18]. Detection using PCR makes it possible to establish the fact of infection of a hive by examining not only infected bees or their excrement, but also the produced honey, in which parasite spores that remain infective are

also found [60, 61]. A recently developed ultra-fast protocol for detecting *N. ceranae* using real-time PCR allows detection of bee infection at the stage of 24 parasite cells in the entire insect body, whereas microscopic detection is usually effective at the stage of mass sporogony, when tens of thousands of *N. ceranae* cells are formed [62]. In addition, molecular diagnostics using PCR makes it possible to assess the degree of infection 8 times more accurately than counting spores during microcopying. Recently, another method for diagnosing nosematosis has been developed, similar to PCR, but not requiring the use of stationary laboratory equipment, which is based on loop-mediated iso-thermal amplification (LAMP) [63].

Prevention and treatment of Asian nosematosis in beekeeping. Recent works by scientists from Italy and Spain have formulated the basic principles of optimal beekeeping to minimize the risks of various diseases and maintain the health of bees in apiaries [64, 65]. In our opinion, these works present the most relevant recommendations to date.

Preventive measures against nosematosis include purchasing queens from bee families not infected with *N. ceranae*, collecting forager bees or debris from the hive in early autumn or spring to diagnose nosematosis microscopically and by PCR analysis. In autumn and spring, bees should be fed stimulants or feed additives to improve their health. Feeding honey and pollen from colonies infected with *N. ceranae* should be avoided. Disinfection of equipment before use is obligatory. The metal tools are sterilized by burning. Fumigation with glacial acetic acid, 5% sodium hydroxide (caustic soda), 0.5% sodium hypochlorite (bleach) and 1.65% ammonia solution can be used to disinfect hives. Queens must be replaced at least every 2 years, except for those that have high genetic value. New families should be kept separately for at least 1 month to control for diseases and infestations and prevent their transmission. Annual renewal of 30% of the cells in the hive is recommended. Beekeepers must minimize stress in bees, winter inspections of hives should be avoided, the use of a smoker should be limited, proper feeding of bees is necessary, etc. IN case of a bee colony death, the hive must be immediately removed from the apiary.

The most effective treatment for both classical and Asian nosematosis is the antibiotic fumagillin, isolated from the fungus Aspergillus fumigatus and which can significantly reduce the infection of the colony and the risk of its destruction [66-68]. Depending on the geographic location and condition of the colony, it is recommended to treat infected colonies from once (in the fall during feeding) to twice a year (in the fall and spring, in case of severe infections). While the fall treatment is aimed at keeping the colony alive during the cold season, the spring treatment is done to improve the health of adult bees, which will be able to properly care for the next generation of individuals raised in the spring. Nevertheless, there are cases where, with an extremely high degree of infection of a bee colony with Asian nosematosis, the use of fumagillin did not stop the spread of infection and did not increase the survival of colonies in winter, regardless of the dose or method of treatment [69, 70]. Cases of resistance to this antibiotic have also been described for *N. ceranae* [68, 71]. In addition, it is important to note that fumagillin cannot be considered completely safe for humans, and the discovery of its residues in honey after processing hives led to a ban on the use of the drug in Europe, and in 2018 the Canadian company Medivet Pharmaceuticals Ltd. stopped its production [72].

Recently, a significant number of studies have been devoted to the search for treatments for nosematosis in bees (Table).

**Studies aimed at finding new treatments for Asian nosematosis caused by the microsporidia *Nosema ceranae* in *Apis mellifera* bees** (2021)

| Substance, method | Effect | References |
|---|---|---|
| Dietary supplement containing wheat bran, essential oils, cinnamon, dextrose, brewer's yeast, lecithin, saturated and unsaturated fatty acids, vegetable proteins, essential amino acids, lipids and a vitamin-mineral complex based on vitamin B | Statistically significant reduction in colony infection with nosematosis by approximately 10% | [73] |
| Various combinations of extracts from 7 types of medicinal plants | A mixture of extracts of 20% blueberry, 40% wormwood, 10% oakmoss, 10% oregano, 10% hops, 5% bay leaf and 5% anise hyssop was most effective against N. ceranae and resulted in more than 2 -fold reduction in infectious load in infected bees | [74] |
| 12 various medicinal extracts plants | 9 out of 12 extracts suppressed the development of infection, the production of N. ceranae spores in infected bees decreased by 4-6 times | [75] |
| Extract of *Agaricus bisporus* | Addition of the extract increased the survival rate of infected bees by approximately 10% and reduced spore production in infected bees by a third. The immunostimulating effect of the extract has been shown to increase the expression of genes encoding abecin, hymenoptaecin, apidecin and vitellogenin in infected bees. | [76] |
| Propolis produced by *A. mellifera* and *Tetrigona apicalis* | Propolis of both types of reduced bee mortality by more than 2 times, infection by 20-40%, infectivity by 70-80% compared to the indicators in untreated bees and led to a significantly higher protein content in the hypopharyngeal glands and hemolymph in treated bees than in untreated bees | [77] |
| Prebiotics from dietary fiber, acacia gum, inulin and fructo-oligosaccharide, as well as commercial probiotics Vetapharm, protexin concentrate with one bacterial strain (*Enterococcus faecium*) and protectin concentrate with several strains of bacteria (*Lactobacillus acidophilus, L. plantarum*, *L. rhamnosus*, *L. delbrueckii*, *Bifidobacterium bifidum*, *Streptococcus salivarius* and *E. faecium*) | Acacia gum caused the greatest reduction in *N. ceranae* spore counts (67%) but also significantly increased bee mortality (62.2%). The *E. faecium* strain produced a similar reduction in spore counts (59%) without affecting mortality. The use of a single strain appears promising as it may reduce the proliferation of *N. ceranae* and increase the survival of infected bees even compared to healthy, uninfected bees | [78] |
| Probiotics eugenol, chitosan, naringenin, protexin | Treatments with eugenol, naringenin and protectin significantly reduced N. ceranae infestation and increased honey production. Protek-sin also increased the number of adult bees, but chitosan was ineffective | [79] |
| Meal from *Brassica nigra* and *Eruca sativa* seeds, containing a fixed quantity various glucosinolates | More than 2-fold reduction in bee infestation in laboratory conditions | [80] |
| Meal from *Brassica nigra* and *Eruca sativa* seeds, containing a fixed quantity various glucosinolates | Reduced infestation of bees compared to control in field conditions, manifested to a lesser extent than when using this technique in the laboratory | [81] |
| Chitosan and peptidoglycan | Stimulation of bee immune responses, increased expression of antimicrobial peptide genes, more than 2-fold reduction in microsporidia infection | [82] |
| Screening of some plant extracts, microbial fermentation products, organic acids, food chain wastes, bacteriocins and fungi | Some of the introduced ingredients such as high concentration acetic acid, p-coumaric acid and Saccharomyces sp. strain KIA1, showed relative effectiveness in the fight against nosematosis | [83] |
| The A. mellifera gene encoding the iron ion transporter transferrin was knocked down | Reduced transcriptional activity in N. ceranae cells, reduced iron loss, enhanced immunity and improved survival of infected bees | [84] |
| Double-stranded RNA, complementary to regions of N. ceranae genes encoding spore coat proteins | More than 2-fold reduction in infestation and increased survival of bees in laboratory conditions | [85] |
| Proteasome inhibitors - ixazomib and ixazomib citrate | Significant reduction in bee infestation and increase in their survival, comparable in effectiveness to the effect of fumagillin | [86] |

N o t e. Unless otherwise stated, the experimental procedure consisted of adding the active substance when feeding infected bees.

Thus, Asian nosematosis of honey bees, caused by an obligate intracellular parasite, the microsporidia Nosema ceranae, is distributed throughout the world.

The infection leads to intestinal dysfunction, hormonal imbalance and energy stress. These factors can change the behavior of infected insects, disrupting the natural division of responsibilities between bees of different ages in the hive. The culmination of such effects can ultimately lead to the sudden death of the colony. Diagnosis of Asian nosematosis usually involves microscopic analysis of insect intestinal preparations and subsequent molecular analysis using PCR or its analogues. The most effective treatment for the disease is the antibiotic fumagillin, which, however, is toxic to humans and is banned in many countries. Special attention is currently being paid to the search for new methods of treating nosematosis, but so far, no safe and effective alternative to fumagillin has been found.

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