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Review

Nosemosis in Honeybees: A Review Guide on Biology and Diagnostic Methods

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Abstract: *Nosema apis* and *Nosema ceranae* are dangerous parasites of the honey bee (*Apis mellifera*). *N. ceranae* is more pathogenic and, nowadays, more widespread than *N. apis*. There are also cases of mixed infections or infections of only *N. apis*. Both *N. apis* and *N. ceranae* can lead to the weakening or death of *A. mellifera* colonies. It is crucial to make a fast and reliable diagnosis to monitor the disease and to start the correct treatment. Additionally, there is a need for further research on the pathogenicity of *Nosema* spp. and also on their prevalence in different regions of the world. In this paper, we present reliable diagnostic methods for *Nosema* spp. infection in honey bees and list the advantages and disadvantages of each method. We have also included basic information about nosemosis and the majority of diagnostic methods in order to provide a source of knowledge for veterinarians and researchers.

Keywords: *Apis mellifera*; diagnostics; nosemosis

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1. Introduction

Nosemosis (*Nosema* spp. infection) is a dangerous, widespread disease in honey bees (*Apis meliffera*) around the world. Nowadays, we distinguish two significant pathogenic microsporidia species for the honey bee: *Nosema apis* and *Nosema ceranae*. It is worth mentioning that in 2017, in Uganda, a third species, *Nosema neumanii* [1], was discovered, but so far there is no data about the pathogenicity of this species. Additionally, its presence outside of Uganda has not yet been confirmed. *N. ceranae* infection is still a challenge for scientists as it weakens colonies, often leading to their death. Moreover, the infection spreads very fast in new regions, possibly displacing *N. apis* infections [2–4]. There are reports of high colony mortality caused by both *N. apis* and *N. ceranae* [5–7]. Recent health status monitoring studies in European apiaries [8–10] show that *N. ceranae* definitely occurs more often than *N. apis*, but mixed infections are also present. There are many methodologies used in the diagnosis and study of *Nosema* infections, including both qualitative and quantitative approaches. This review guide may be a useful tool for researchers and veterinarians as it presents the most important basic information on *Nosema* biology, the most commonly used methods in diagnostic and research areas, and potential uses for these methods. We also present the advantages and disadvantages of these methods, which have been developed mostly in Europe, where nosemosis is widely prevalent [2]. Although the number of studies on nosemosis is constantly growing, and the methods of identification for each species are still being improved to make results faster, easier to obtain, and more accurate, it is sometimes difficult to decide which method is best for a particular type of research. This review aims to present the most useful methods in a concise and systematic way in order to make the choice easier and more informed by adding crucial information about the biology and pathogenicity of this Microsporidium.

2. *Nosema* Life Cycle and Spread

Microsporidia *N. apis* and *N. ceranae* are pathogens of the epithelium of the honey bee midgut. The parasite develops in all the castes of the bee colony (workers, drones, and queens).

In the external environment, these microsporidia exist in the form of spores, which must be eaten (with food or water) by the bee in order for the infection to start [11]. The mature spore starts to germinate in the midgut lumen by extruding its polar filament. The spore requires a change in pH to activate the process of polar filament ejection. The polar filament penetrates the cell lumen, and the sporoplasm (the inside of the spore) flows through it into the host cell [12]. In the host cell, the sporoplasm transforms into a meront and starts to multiply (by division) into sporonts. After proliferation, sporonts transform into sporoblasts, which have a thick cellular wall. Sporoblasts transform into mature spores that pass into the lumen of the host gut [13]. Spores of *N. apis* and *N. ceranae* can pass from one epithelial cell to another and still reproduce, which contributes to their high pathogenicity [14,15]. At the end of the process, the spores are passed into the rectum and exit in the feces (which are the main source of *Nosema* infection) [11,13]. Since the spores destroy the gut lumen, the bee cannot ingest or digest food. Therefore, there are considerable amounts of (undigested) sugar in the feces (which is attractive to other bees), and bees lick and eat it (the oral–fecal route of infection). Combs contaminated with feces are the basic source of infection [16]. *Nosema* spores also spread through honey, pollen, bee bread, syrup, and other routes, thus trophallaxis (oral–oral food exchange between insects) is a direct way of sharing the spores between bees (Figure 1) [17,18]. It causes dissemination of the spores through all the castes of the colony (including the queen) [19]. A study by Roberts and Hughes [20] shows that drones can be 'superspreaders' of *N. ceranae* spores through trophallaxis.

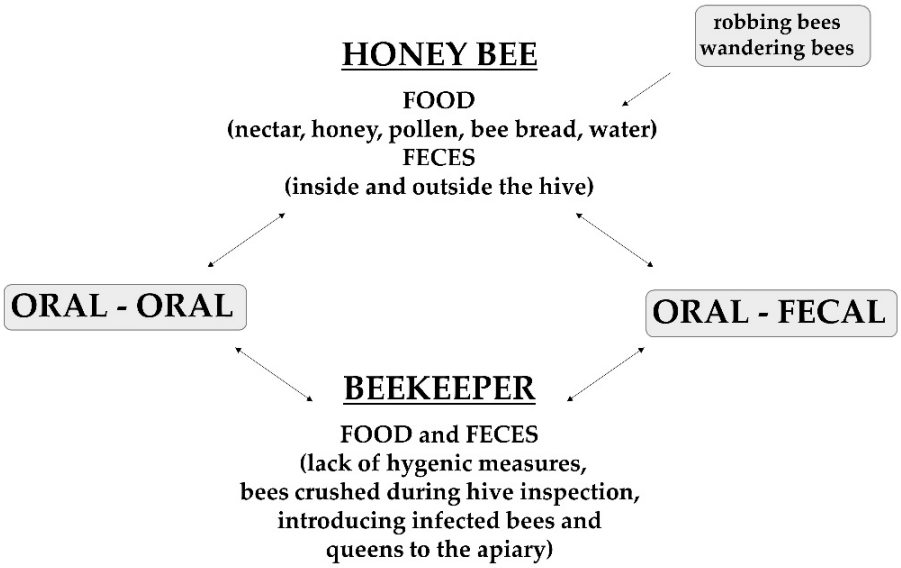

Figure 1. Routes of *Nosema* spp. infection and spread.

The complete life cycle of *N. apis* can last about 5 days [11]. In cage trials, the life cycle of *N. ceranae* lasted 3 days [15]. Some studies show that when a 100% infectious dose (114×10^3 spores per bee) is used, the youngest (newly emerged) bees are the most susceptible to the infection [21], while other studies suggest that, with the same infectious dose, 5-day-old bees are the most susceptible [22]. It has been proven that *N. ceranae* can infect basal cells in the midgut while *N. apis* cannot [15]. Epithelial cells in the midgut exfoliate constantly, so the basal cells proliferate and replace them; thus, when basal cells are infected, the intestinal wall is damaged irreversibly, which points to the higher pathogenicity of *N. ceranae* in comparison with *N. apis*. Other in vivo studies have also

confirmed the higher pathogenicity of *N. ceranae*. For instance, Forsgren and Fries [23] showed that the average infectious dose of *N. ceranae* spores was over 4.5 times lower than of that of *N. apis*, and it possessed an LD50 of about 85 spores. However, in the United States, the results were very different. Huang et al. [22] showed that the infectivity of *N. apis* was higher than *N. ceranae* (for bees of the same age and the same infectious dose). The contradictory results may be due to the different climatic conditions from which the bees originated or even a genetic component; for instance, Danish bees are resistant to *Nosema* infections (in a long-lasting breeding program) and the pathogenicity of this microsporidium is minor there [24]. It may also be due to a black queen cell virus infection, which is known to aggravate the course of nosemosis significantly and which is not checked for in most *Nosema* studies [25].

Both *Nosema* species exhibit different temperature tolerances. *N. ceranae* is very sensitive to freezing, while *N. apis* can be stored in a freezer without losing much vitality [16]. However, in contrast to *N. apis*, *N. ceranae* can survive above 60 °C [26]. Presumably these physical factors determine the prevalence of *Nosema* infections. Therefore, a majority *of N. ceranae*-infected colonies are found in warmer climates [6,10] and *N. apis* in colder climates [27]. During recent years, it has been observed that *N. ceranae* is displacing *N. apis* [2]. There have been many cases of increased collapses of bee colonies caused by *N. ceranae*, mostly in the Mediterranean countries (warmer climate) [2]. In a moderate climate, colony losses due to *N. ceranae* can also be quite severe. For example, winter colony losses in 2007/2008 in Poland were mainly due to type C nosemosis, and this problem occurs in Poland periodically, every few years [7]. This displacement of one species by another is probably connected to climate change [28]. Furthermore, in Europe, the prevalence of mixed infections is rather low (less than 10% of samples) [3,8,9]. However, in studies outside of Europe, in a moderate climate, the high pathogenicity of *N. ceranae* has not been confirmed [22] and, for example, in Asia [4], the prevalence of co-infection is more than 35%.

3. Course of Infection

3.1. Type A Nosemosis

During *N. apis* infections (called nosemosis type "A") there is a strong seasonality. Forager bees bring the parasite to the hive (with infected pollen, nectar, or robbed food stores). During the summer, the disease develops slowly and there are usually no symptoms. Young bees and brood remain uninfected as *Nosema* spp. does not infect larvae [11]. The slow progress of the disease in the summer is also due to the sensitivity of *N. apis* spores to high temperature [26]. During the autumn, there are more older bees in the colony compared to bees of younger generations, so more bees are infected (in comparison to the summer). In addition, autumn bees live longer than summer bees, so spores have more time to multiply to greater numbers. Then, in the winter cluster, they infect the rest of the bees. If the colony survives the winter, there is usually an outbreak of the disease in early spring [11,29].

At the individual level, infection with *N. apis* influences mostly workers. Epithelial gut cells lyse and the secretion of digestion enzymes decreases [30]. Atrophy occurs in the hypopharyngeal glands of nurse bees [31,32], which leads to a decrease in royal jelly production, thereby reducing brood rearing. Because of epithelial cell degeneration, the digestion and absorption of nutrients become severely impaired, the bees become malnourished, and often "die of starvation" while eating considerably more. As a consequence of almost no absorption of nutrients, there are not enough proteins to build a fat body [30].

Because food is not ingested properly, diarrhea can be observed (see Table 1). The front wall of the hive, frames, and the entrance are often contaminated with brown feces [11]. Adult bees have extended (swollen) abdomens, and they crawl around the hives and hive entrances as they are unable to fly. Infection with *N. apis* causes a faster transition of nurse bees into guardian bees and foragers (which means that those insects age faster) [33] but surprisingly does not reduce bee lifespans significantly [34]. However, the number of

bees decreases, spring development of the colony is inhibited, and honey production is reduced [30]. In extreme cases, the colony dies during the winter or early spring [35]. Significant damage to yolk granules and the degeneration of the ovaries can be observed in infected queens [36]. Additionally, studies show that *N. apis* infection has a negative influence on the effectiveness of sperm transfer to the queen's spermatheca [37]. In addition, during the spring, when the infected queen lays a smaller number of eggs or does not lay at all, she is usually replaced by supersedure [36].

Table 1. Symptoms of both type of nosemosis which can be observed in the colony.

Type of *Nosema* spp.	Symptoms	Time of the Season
Nosema apis	Fecal streaks present on combs and hive exterior Dead bees at the hive entrance Swollen abdomens in adult bees Diarrhea in adult bees Crawling bees	Early spring
Nosema ceranae	Symptomless ("silent killer"): 4 phases of infection (see Section 3.2) Death of the colony (handful of bees with the queen)	Autumn or early winter

3.2. Type C Nosemosis

Cells attacked by *N. ceranae* (which causes nosemosis type "C"), similar to type A nosemosis, are lysed and degenerated [15]. During the infection, the midgut and basal cells are severely damaged. Atrophy of the hypopharyngeal glands is observed (less royal jelly). Additionally, the secretion of hormones becomes impaired. Decreased levels of vitellogenin (Vg, a protein important in reproduction and the social life of bees) and increased levels of juvenile hormone (JH, regulation of vitellogenesis) in nurse bees [38] have an extremely negative impact on brood rearing. Changes in the Vg–JH feedback loop cause infected bees to foraging significantly earlier than the non-infected ones (fewer nurse bees). Decreased secretion of Vg results in oxidative stress [38], which causes unrestrained hunger (energetic stress) [39]. That hunger causes an increase in the occurrence of trophallaxis events and probably leads to the shortened lifespan of the bees [39]. Infected bees die more often in the field while foraging, likely due to altered flight ability and impaired memory [35]. In cage trials, the lifespan was no longer than 8 days post infection, independently of the dose of spores [15]. Additionally, all of the above processes cause immunosuppression [18].

The disease caused by *N. ceranae* is called dry nosemosis because, during the course of infection, diarrhea is not observed [6,15]. *N. ceranae* infection is more widespread in foragers than in hive bees, but, in fact, the queen is also exposed. In highly infected colonies, supersedure is reported more often than in healthy colonies [35].

There are no characteristic symptoms that may be simple to notice by the beekeeper. The four phases of the *N. ceranae* infection in the colony were described in [6,40]. The first phase is from spring to early autumn. It is entirely symptomless, and the number of spores per bee is lower than 10^6. In autumn and winter, a normalization of strength in the colony is observed (phase 2). The queen continues to lay eggs and to hold the number of bees at appropriate levels. The percentage of infected foragers increases ($\geq$65%) because the majority of bees in the cluster are adult (the parasite has had time to reproduce). In the next season (the following spring), we observe the so-called "false recovery", phase 3. The colony looks as if it is very strong as the number of bees is very high, and almost all frames are full of brood, but still, there is usually no swarming. The percentage of infected foragers is <65% because a lot of young bees that are not yet infected are present. The fourth phase is depopulation. It occurs in autumn or early winter. Sometimes colony death occurs in early spring. Only a handful of bees, with the queen, some food, and brood remain in the hive. In that phase, the percentage of infected bees is again $\geq$65% [6,40].

It is worth mentioning that *N. ceranae* infection becomes more severe when the bees are simultaneously exposed to neonicotinoids [2]. Black queen cell virus, which often co-occurs with *N. ceranae*, also significantly aggravates nosemosis "C" [25,41].

Due to such big differences in the pathogenicity of "A" and "C" nosemosis, it is crucial for both beekeepers and scientists to know exactly which pathogen they are dealing with. There are many diagnostic methods to determine both the severity of the infection and the identification of the *Nosema* species. We describe the most relevant and often used methods below in order to help both diagnosticians and researchers choose the proper one.

4. Bees Used in Diagnostic Methods

To test for nosemosis, forager bees are used as they are the most likely ones to contain large spore numbers. The World Health Organization recommends collecting bees from edge frames or forager bees returning from the field.

The optimal number is 60 bees [26]. Bees should be killed humanely, through freezing or by using carbon dioxide [42].

5. Diagnostic Methods

5.1. Light Microscopy

Microscopic examination can be used as a basic, preliminary method to check whether *Nosema* spp. spores are present in the sample (Table 2). It is basic method for diagnosticians and veterinarians. The abdomens of insects are separated and ground up with a mortar and pestle with 5 mL of water (after that, water is added to obtain 1 mL per bee) [26]. A drop of solution is put on a slide, covered with cover-slip, and examined microscopically at 400× magnification. A bright-field or phase-contrast microscope can be used. Spores of *Nosema* spp. are oval with a dark edge. The size of *N. apis* spores is about 4–6 µm × 2–4 µm [13] and *N. ceranae* 4–4.8 µm × 2.1–2.9 µm [18]. The spores of *N. apis* are rounder and slightly larger in comparison to the slender, oval spores of *N. ceranae*. Experienced scientists can roughly distinguish between species using microscopy, but this is not a recommended method as some portions of spores of both species may look very similar, which leads to misdiagnosis. There may be too many errors here, especially since mixed invasions occur [43].

Microscopy is also used as a quantitative test to determine the level of infection (expressed as the number of spores per bee). It requires the use of a haemocytometer. The OIE method [26] uses the Neubauer hemocytometer, and for the purpose of this study, we describe the process with the use of this counting chamber. To begin, 60 abdomens of workers were ground up in a mortar with 5 mL of water. In the next step, 50 mL water was added and mixed. The solution was filtered through two layers of muslin (cotton fiber). Mortar and pestle were flushed with 5 mL water and that water was also filtered. When the solution was homogeneous (acquired by thorough mixing of the filtrate directly before the next step), one drop was put on the hemocytometer chamber. All spores on the grid were counted. The result is calculated according to the formula:

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

Z = spore number per bee
α = total number of spores counted
β = number of squares counted
δ = dilution factor

The spores can be stained to help distinguish them from other organisms or artefacts, e.g., with Giemza staining [44]. Material embedded on a glass slide can be stained with toluidine, which also helps to see the spores in a qualitative method [45].

Table 2. Methods used in *Nosema* spp. diagnostics with their key components, advantages, and disadvantages.

Method	Result	Equipment Required	Reagents	Primers (If Applicable)	Advantages	Disadvantages	References
Light microscopy	Quantitative	Mortar and pestle Light microscope	Water	n/a	Simple, fast, low cost	No species determination	[26]
Electron microscopy	Qualitative	Electron microscope	2% Glutaraldehyde 2.5% Paraformaldehyde PBS pH 7.4 1% Osmium tetroxide Acetone Resin Dye	n/a	Species determination	Expensive, time-consuming	[1,15,43,46]
End point PCR	Qualitative	Thermocycler	ddH_2O Liquid nitrogen DNeasy® Plant Mini kit (Qiagen) (A) or High Pure PCR(Roche Diagnostic) (B) DNA template Primers	Muniv Rev (A) Mnceranae Fwd (A) Mnapis Fwd (A) or UnivRev (B) NaFor (B) NcFor (B)	Species determination, sensitive	Equipment needed, expensive	[3,5,44,47–49]
		UV-transilluminator	Agarose gel Dye				
qPCR [1]	Quantitative, qualitative	Real-time PCR Thermocycler	DNA template SsoFast EvaGreen® Supermix DdH_2O Primers	NaFor NcFor UnivRev	Efficient, fast, sensitive	Expensive, equipment needed	[21,44,50–53]

Table 2. *Cont.*

Method	Result	Equipment Required	Reagents	Primers (If Applicable)	Advantages	Disadvantages	References
HBRC method [2]	Quantitative	Thermocycler	Hexadecyl trimethyl ammonium bromide		Inexpensive, sensitive	Equipment needed	[52]
			Tris hydroxymethyl aminomethane				
			Methylene diamine tetra-acetic acid				
			NaCl				
			ddH_2O				
			Proteinase K solution				
			Phenol				
			Chloroform				
			Primers	MITOC-F			
				MITOC-R			
				APIS-F			
				APIS-R			
		UV-transilluminator	NaOAc				
			Ethanol				
PCR-RFLP [2]	Qualitative	Thermocycler	DNA template		Fast, accurate, species determination	Expensive, equipment needed	[42,54,55]
			Endonucleases				
			Buffer Nebbuffer				
			Primers	SSU-res-f1/r1			
				30 SNPs/27 SNPs			
				3 INDELS/11 INDELS			
				16 SNPs			
				8 INDELS			
		UV-transilluminator	Agarose gel				
			Dye				
LAMP [2]	Qualitative	UV-transilluminator	DNA template		Fast, sensitive		[56–58]
			DNA polymerase buffer				
			Dye (HNB)				
			Betaine				
			dNTP				
			DNA polymerase large fragment				
			Primers	cerFIP			
				cerBIP			
				cerF3			
				cer B3			
				cerLF			
				cerLB			
				apFIP			
				apBIP			
				apF3			
				apB3			
				apLF			
				apLB			

Table 2. *Cont.*

Method	Result	Equipment Required	Reagents	Primers (If Applicable)	Advantages	Disadvantages	References
Immuno-diffusion	Qualitative	Microplates	ddH_2O	n/a	Fast, simple, low cost	Only detects *N. ceranae*	[59–61]
			ELISA kit for rabbit primary antibodies				
			TMB				
		Microplate reader	Stop solution				
Fluorescence	Qualitative	Fluorescence microscope	PBS	n/a	Reliable, distinguishes dead/living spores	No species determination	[62–64]
			Sytox green				
			ddH_2O				
			DAPI				
Chromato-graphy	Qualitative	Dryer (Speedvac)	Hemolymph	n/a	Reliable, useful in research	Expensive, equipment needed	[65,66]
			Methanol-ethyl acetate				
		Gas chromatograph	Methoxylamine hydrochloride solution				
			MSTFA				

[1] Preparation of DNA the same as for end-point PCR. [2] PCR amplification the same as for end-point PCR.

5.2. Electron Microscopy

Electron microscopy is an expensive and time-consuming method (Table 2). It is used in research as a tool in tissue pathology, including to confirm tissue tropism [67]. This method has no use in confirming infection but possibly can be used in distinguishing between species. Species distinction is based on the number of filament coils in the spore [68]. In *N. apis* spores, there are 26–32 filament coils [69], while in *N. ceranae* spores, there are 18–23 [68]. Spores of *N. apis* are bigger than *N. ceranae* (see light microscopy paragraph) [44].

The tissues for TEM (transmission electron microscopy) are prefixed in 2% glutaraldehyde/ 2.5% paraformaldehyde for 1 h and then washed 3 times in phosphate buffer pH 7.4 [15,46]. Then, 1% osmium tetroxide at room temperature is used to post-fix the samples. Specimens should be washed in phosphate buffer, dehydrated with an ascending acetone series, and embedded in Epon–Araldite resin [46] or Spurr's resin [1,43], according to Higes et al. [15]. Epon block is trimmed for ultrathin sectioning and double stained with 2% uranyl-acetate and lead citrate Reynolds solution for 10 min each. To mount the tissue on the microscope table Formvar-coated grid can be used [1]. In SEM (scanning electron microscopy), samples are not stained but mounted on specimen stubs (using adhesive tape) and coated with gold [70].

5.3. Molecular Diagnostics

5.3.1. Endpoint PCR

As conventional microscopy is rather imperfect in distinguishing *Nosema* species, and electron microscopy requires a lot of work, a polymerase chain reaction (PCR) method was developed as a very specific tool to distinguish between the species (Table 2). Later on, a real-time PCR (qPCR) method was developed as a quantitative molecular tool.

In research, mainly multiplex-PCR, PCR-RFLP, and qPCR are used. The first attempt of PCR reaction to distinguish *Nosema* species was in 1996, and the product of this reaction was sequenced and placed in Gene Bank (accession number U26533) [32,68].

The BEEBOOK is a practical manual of standard methods for the honey bee. The goal of this project is to standardize honey bee research methods around the world so that test results from different laboratories are comparable and reliable. The fact that all the methods described in the BEEBOOK have been validated by leading honeybee experts is the reason we used it in the review.

The first step in all polymerase chain reaction methods is DNA extraction from insect tissues, the best from all bees in a sample (homogenate). Fries et al., in BEEBOOK [44], recommended using 30 bees and mixing with 15 mL double-distilled water (ddH_2O) (0.5 mL/bee) in a one-to-one ratio. 100 µL of the homogenate is centrifuged for 3 min at 16,100 g. The obtained pellet contains microsporidia and other cellular material. The pellet should then be frozen in liquid nitrogen and crushed to open spore walls. Alternatively, another method can be used. It is based on spore germination rather than mechanically breaking their walls, but it takes significantly more time [5].

Usually, to carry on DNA extraction commercial kits, such as DNeasy® Plant Mini kit (Qiagen) [44] or High Pure PCR template preparation kit (Roche Diagnostic) [5,47], are used. The PCR reaction mixture recommended by the BEEBOOK [44] contains 1 µL DNA, 0.5 U Taq polymerase, 2× Taq reaction buffer, 3 mM $MgCl_2$, 0.3 mM each dNTP, 0.4 µM for each primer for *N. apis* and *N. ceranae*, 0.5 µM for each primer for *N. bombi* (as it is a multiplex reaction for *N. apis*, *N. ceranae*, and *N. bombi*), and Muniv-R completed with PCR-grade water to 10 µL of mixture volume. Recommended primers are [44]:

For *N. apis* Mnapis-F: 5′-GCATGTCTTTGACGTACTATG-3′
For *N. ceranae* Mnceranae-F: 5′-CGTTAAAGTGTAGATAAGATGTT-3′
For *N. bombi* Mnbombi-F: 5′-TTTATTTTATGTRYACMGCA-3′
Muniv-R: 5′-GACTTAGTAGCCGTCTCTC-3′

The amplification conditions are as follows [44]: initial denaturation step of 95 °C for 2 min, next 35 cycles (parameters: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s), and final extension step of 72 °C for 5 min. Visualization of products is performed by electrophoresis in 2% agarose gel with ethidium bromide or other stains such as GelRed. PCR product sizes are as follows:

For *N. apis*: 224 bp
For *N. ceranae* 143 bp
For *N. bombi* 171 bp

It is recommended to use primers designed based on 16S rRNA smaller subunit gene as the most reliable method [26]. Many researchers design primers independently and amplify the DNA based on them [5,47,48]; others use Gene Bank [3,49].

5.3.2. Quantitative PCR (qPCR, Real-Time PCR)

Quantitative real-time PCR allows for both relative quantification and absolute quantification of *N. apis* and *N. ceranae* spores in a sample. The precise information to carry out the reaction is described by Fries et al. [44]. The first step is the design of primers which should be species-specific. Usually, the qPCR reaction mixture is created separately for *N. apis* and *N. ceranae* using dedicated primers (NaFor and NcFor respectively). Mastermix contains 2 µL DNA, SsoFast EvaGreen® Supermix, 0.4 µM of dedicated primer, 0.4 µM UnivRev primer, and 6.4 µL water. The reaction is carried out under the following conditions: initial enzyme activation step for 15 min at 98 °C and 40 cycles (denaturation at 98 °C for 5 s, extension at 63 °C for 10 s, melt curve analysis from 65–95 °C one step/10 s) [44]. According to other studies, we can contain two tested samples and the necessary reagents in one real-time PCR reaction mixture [50,51]. The normalization of the PCR reaction is crucial to carry out it correctly. The most common way is to compare the DNA level of a sample to a reference gene for which the detection level is generally stable [50,52,53]. The qPCR test seems to be a fast, efficient, and sensitive method for detecting infections of both microsporidia species (Table 2) [51]. That method was also used to determine the infection level of *N. ceranae* depending on the ages of the bees and also depending on the way of infecting (individual or collectively infection) [21].

Another innovative system to quantify *N. ceranae* and *N. apis* in the sample uses the light intensities of bands. Products of amplification are separated by electrophoresis in 1.1% gel, photographed, and developed in a dedicated program [52]. Amplification is performed in a standard thermocycler, not a real-time thermocycler. In the HBRC

(abbreviation of components of buffer) method buffer consists of 0.03 M hexadecyltrimethyl ammonium bromide, 0.05 M tris hydroxymethyl aminomethane, 0.01 methylenediamine tetra-acetic acid, 1.1 M NaCl, and distilled water. In comparison to the germination-extraction method, [5] the HBRC method is quite inexpensive, less time-consuming, and still highly sensitive (Table 2). To obtain relative quantification, genes of both pathogens' hosts were used additionally [52].

Both methods (endpoint PCR and qPCR) may be used by scientists and diagnosticians, but they are more expensive than microscopy.

5.3.3. PCR-RFLP

Polymerase chain reaction—restriction fragment length polymorphism is a reaction using specific enzymes. They digest DNA in species-specific fragments. Products of the reaction are visualized in a gel [32]. Protein sequence analysis is not necessary for RFLP. Primers for the three microsporidian species *N. apis*, *N. ceranae*, and *N. bombi* are designed based on small RNA subunits. The products obtained in the PCR reaction are differentiated due to three different restrictive endonucleases. The reaction mix is 12.5 µL and contains 10 µL amplified DNA and 1.5 U of enzymes (endonucleases). Buffer Nebbuffer 2 is used and the reaction lasts 3 h at permanent temperature 37 °C [42]. The method is quite fast and accurate, but it is still not widely used. Primers from another study were designed on a large RNA subunit and only one enzyme was used to cut DNA. The method is effective and the author recommends it for differential diagnostics [54]. In another study, two different restriction enzymes were used to differentiate *N. apis* from *N. ceranae*: Rsa I and Dra I, respectively [55]. The reaction was carried out overnight at 37 °C. The abovementioned methods have considerable potential for being used as diagnostic tools as well as techniques in research due to their fast performance and relatively low costs (Table 2).

5.3.4. LAMP Method

Loop-mediated isothermal amplification (LAMP) dedicated to detecting both *Nosema* spp. was described by Ptaszyńska and co-workers in 2014 [56]. It is based on isothermal amplification of specific DNA fragments. It differs from standard PCR. The reaction is carried out at a constant temperature and lasts only half an hour. There is a large amount of DNA produced, after introducing magnesium into the sample, with a positive result for the test, and the product can be seen with the naked eye (white sediment at the bottom of the test tube). According to the author, the method is highly specific and about 10^3 times more sensitive than standard PCR. The specificity of this method was also confirmed by other studies, which also showed that the LAMP method offers a lower pathogen threshold of detection rate than conventional PCR [57,58]. Authors from Thailand have developed a method in which 0.3 ng of the tested DNA is required [57]. However, in studies from 2004, 100 fg of the tested DNA was required [56]. Different concentrations of some primers, dye (HNB), betaine, and dNTP were used to obtain the best reaction mixture. Finally, that reaction's components are listed as follows: 1.2 µmol/L FIP primer and BIP primer, 10 µmol/L F3 primer and B3 primer, 0.6 mmol/L dNTP, 0.6 mL/L betaine, 4.8 IU Bst DNA polymerase large fragment, 1 DNA polymerase buffer B, 120 µmol/L dye HNB, and 0.3 ng tested DNA. The mix is incubated at a permanent temperature of 60 °C for 40 min. To finish the reaction, the sample is heated at 80 °C for 2 min. The result of the reaction is visible to the naked eye [57]. Ptaszyńska used six primers in the LAMP reaction, and scientists from Thailand used four [56,57]. In a 2020 study [58], the authors developed a direct LAMP method in which they used purified *N. ceranae* spores instead of DNA extract in reaction mix. As in the previous methods, the dye (1 µL SYBR™ Safe) is added directly to the reaction tube. After UV light exposure the result is immediately visible, and a yellow reaction color means a positive result and red a negative result [58]. Given that the reaction is quite simple to perform and does not require much equipment, it might be suitable in diagnostic and scientific work (Table 2).

5.4. Immunodiffusion

In 2016, researchers created the first serological test for microsporidia *N. ceranae*, which can be used in both laboratory and field conditions [59]. However, this is not a method for differentiating between *Nosema* species but to detect *N. ceranae* only (Table 2), and it can be useful after basic microscopic testing. The researchers developed a new antibody against a spore-wall protein, SWP-32, of *N. ceranae* [60]. The authors used an ELISA kit for rabbit primary antibodies and tested samples according to the instructions for spore suspensions. The results collected by the immunodiffusion method were highly matched to the RT-PCR results from the same samples [59]. According to the authors, this method is cheaper, simpler, and less labor-intensive compared to qRT-PCR. The concentration of spores in the samples is 1×10^3 [59]. Kim and Lee developed three monoclonal antibodies specific to *N. ceranae* spore proteins which can be used in a rapid and accurate diagnostic methods (ELISA and Western blotting tests) [61]. According to the authors, the method has higher specificity than the ELISA kit based on antibodies against SWP-32 [60], and the probability of false positives is very low. The concentration of spores in the samples detected via ELISA was 1×10^4 [61]. The authors plan to develop an ELISA-based rapid detection kit as a diagnostic tool for *N. ceranae*.

5.5. Fluorescence

Fluorescence can be used to detect infection of *Nosema* spp. but not to discriminate species (Table 2). This method allows for distinguishing between dead or living spores of *N. ceranae* [62], making it suitable for scientific research rather than diagnostics. After dual staining with Sytox green and DAPI staining, samples are examined under a fluorescent microscope. Spores are obtained from grinding the gut of honey bees with ddH_2O [62]. The same method was developed with *N. apis* spores, but the authors used SYTO 16 and propidium iodide staining [63]. In other studies, the authors also used dual staining, with Fluorescent Brightener 28 and the DNA dye propidium iodide [64]. However, in this study, the authors used midgut of the honey bee fixed with 3.2% paraformaldehyde [64].

Nosema spore viability was also studied by flow cytometry, which is based on fluorescence intensity. According to the authors, this method proved to be reliable, fast, and better in quantification compared to traditional fluorescent microscopy [63].

5.6. Chromatography

In studies from 2012, researchers used gas chromatography–electron impact mass-spectrometry for metabolite profiling of hemolymph of bees infected by *N. ceranae* [65]. Hemolymph was sampled with extreme care (from a small cut in the thorax, collected by pipette) to avoid contamination of the samples, especially with the hemolymph from the intestine. A matrix of data, which were collected during GC/MS for healthy and infected bees, was developed. It was proven that the metabolic profile of the hemolymph differs in healthy and infected honey bees. The most significant differences were in the levels of the carbohydrates and amino acids, i.e., in infected bees the level of glucose was higher [65]. The presence of *N. ceranae* spores in the examined bees was checked by qPCR.

Other studies have shown that, in honey bees infected with *N. ceranae* (Spanish strain), the level of juvenile hormone increases [66]. However, the concentration of JH in the hemolymph of healthy honey bees was slightly lower in comparison to bees infected with both species of *Nosema*. Chromatography in these methods was a reliable and simple method to use; however, it can be better used for metabolic testing during infection rather than for diagnosis (Table 2).

6. Conclusions

Infections of a bee colony with both *Nosema* species are not new to beekeepers or veterinarians. However, they are still a serious problem. It is extremely important to diagnose and monitor the health status of bees as well as to further research this problem and possibly to find better ways of solving it. This work describes the biology of *Nosema* spp.

as well as the etiopathology of nosemosis to pinpoint areas of importance for both diagnosticians and scientists. It displays the methods used in *Nosema* diagnostics worldwide and highlights their usefulness for research or diagnostic work as well as their advantages and disadvantages. It should prove especially useful for laboratories that only start their work with *Nosema*, as we also show methods that do not require specialist equipment and are cost-effective. We also clearly show which methods are time-consuming and whether they can distinguish between *Nosema* species (a qualitative method) or show the level of infection (a quantitative method), or both. Such comparisons give the reader a clear picture and make the choice of methods easier and based on financial, time, and equipment availability factors.

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