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Partial Detection of *Nosema Ceranae* in Honeybees *Apis mellifera* L. and Molecular Characterization of Mitochondrial Cytochrome Oxidase I Gene

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Abstract

Honey beekeeping is a large-scale global industry in the world; bee health suffers from one of the most dangerous diseases by *Vairimorpha* (*Nosema*) pathogen. The molecular genetic diversity among the collected honeybee populations was investigated using the mitochondrial DNA cytochrome oxidase subunit I (COI). To determine the level of *Vairimorpha* (*Nosema*) ceranae (*V. ceranae*) concentration within honeybee individuals in the colony, we dissected the honeybee and extracted the midgut, ovary from newly hatched virgin queens, and hypopharengeal gland and midgut from newly hatch worker bees. The spores were counted initially using light microscopy. The quality and quantity were determined by conventional PCR and qPCR technology to track infection levels. The sequence of COI showed that belong to *Apis mellifera* Arabia haplotype by 99% and by 78% to African haplotype *Apis mellifera capensis*. *Vairimorpha ceranae* with significant differences ranged from 0.90×10^6 spore/bee to 18.0×10^6 spore/bee (samples code C7) for adult bees. While the copies number of *V. ceranae* varied from one organ to another, the pathogen was in newly hatched workers or virgin's queens higher than old generation. The hypopharengeal glands in new hatch honeybees' workers were the most infected of all organs by 25.49×10^6 copies. These results demonstrate the importance of maintain proper quarantine measures to reduce infection. Also, these results could explain how *V. ceranae* pathogen is transmitted vertically and horizontally within and between the colonies.

Keywords: *Apis mellifera*, COI, *Nosema ceranae*, RT-PCR, Microscopy examination

Introduction

Microsporidia are a group of intracellular eukaryotic microorganisms that are closely related to the Fungi; Honeybees are eusocial insects that intimately interact within densely populated colonies, creating a suitable environment for pathogens, including microsporidia (Zhang et al., 2021). The decline number of healthy honeybee colonies has a negative influence on honey production as well as on agricultural crops and ecological plants due to the decrease in pollination in flowering plants (Gallai et al., 2009; Glenny et al., 2017). There are two common microsporidian species; *Vairirmorpha* (*Nosema*) *apis* and *Vairimorpha* (*Nosema*) *ceranae* (genus recently redefined by Tokarev et al., 2020) identified in European honeybees (*Apis mellifera*).

Nosema ceranae (*N. ceranae*) is the most common gut pathogen in adult honeybees (Chen et al., 2009) and was detected in all members of honeybees' colony, including adult bees and brood stages (Truong et al., 2021). Chronic infections are commonly described for microsporidian species (Solter, 2014). *Nosema ceranae* infections mainly occurred in midgut epithelium cells and have characteristics of typical chronic infections (Holt et al., 2013), including hormonal and immune responses (Badaoui et al., 2017) and subtle alterations of behavior (Wolf et al., 2014). Another main organ was recorded as infected with *Nosema ceranae* is hypopharengeal glands (HPGs) tissues in infected honeybees (Chen et al., 2009); and Alaux et al. (2010) demonstrated that Nosema infection induces gland atrophy, which led to less HPGs activity and a lower protein content (Corby-Harris et al., 2016; Li et al., 2019). The HPGs, located in the anterior area of the heads of worker notions, are responsible for concealing protein-rich food royal jelly which is used to feed all larvae and queen of colonies (Winston, 1991; Hrassnigg and Crailsheim 1998). Therefore, any detrimental impact on the development of the hypopharyngeal glands could significantly affect brood rearing and ultimately the colony growth (Jack et al., 2016).

Unfortunately, *Nosema ceranae* was detected in previous studies in immature developing queens and possibly to drones (Higes et al., 2009). Although, Traver and Fell (2012) investigated *Nosema ceranae* infection in the ovaries, however, there is no evidence of the pathogen transmission from the queen to her eggs according to Roberts et al. (2015) and Goblirsch (2018).

This pathogen can be detected using a microscope and is one of the standard methods for detecting and quantifying Nosema (Fries et al., 2013). But microscopic examination is not an effective way to detect rhinitis at a low-level Severity of infection (Truong et al., 2021). So, Polymerase chain reaction (PCR) detection has been commonly used as a reliable method for diagnosing Nosema infection (Nosemosis) (Rivière et al., 2013) and quantitative real-time PCR was developed for accurate and sensitive detection of and *Nosema ceranae* simultaneously, using multiple primers in one reaction (Forsgren and Fries, 2010; Fries et al., 2013). Since, quantitative PCR is used as a potent tool for detecting and measuring the level of *Nosema ceranae* infections as well as for related studies, such as the evaluation of virulence of Nosema species (Forsgren & Fries, 2010).

Accordingly, the current study aimed to confirm between the traditional microscope detection method and the molecular detection of Nosema within infected colonies. Therefore, the most important organs of the newly hatched workers, such as the hypopharengeal glands and midgut, as well as the newly hatched virgin queen's midgut and ovaries, were chosen to detect Nosema; an attempt to determine the correlation in the transmission of the pathogen between the individuals of a single colony, horizontally or vertically.

Materials and methods

Honeybee samples collection

The samples studied were collected in the summer 2023 from the apiary at Bee Research Department, PPRI, ARC. Ten pools of adult hybrid honeybee forager workers samples from each infected colony under study were collected randomly and divided into four groups and each group has 3 replicate (G1, G2, G4 and G7) and noticed the colonies in group no.7 (G7) were more collapsed so, we used it as a standard infection of *Vairimorpha* (*Nosema*) *ceranae*. The samples were divided for microscopical investigation (taken 4 out of ten members) and for qPCR tests (taken 6 members out of ten).

Nosema ceranae spores' detection microscopically

Four honeybees out ten from the studied samples for each marking colony separately and examined the *Vairimorpha (Nosema) ceranae* spores under microscope according to Cantwell (1970) using a light microscope (ML 2000, Japan). The spores of *Nosema ceranae* were counted using a hemocytometer slide (Paul Marienfeld GmbH, Germany) for each one. On the other hand, RT-PCR to using too to detect spores of *Nosema ceranae*.

DNA Extraction

Honeybee worker legs were used for DNA extraction (ten pooled) and continued by identification of cytochrome oxidase subunit I gene (COI), and six adult workers honeybees from studied colony (No. 7 (C7)) as a standard sample for *Nosema ceranae* using QI Aamp mini elute spin kit (Qiagen, GmnH, Germany, cat no. 51304).

Oligonucleotides synthesis

Cytochrome c oxidase I gene (COI) was amplified by using primers: LCO1490 Fwd. (5-′ TTTCAACWAATCATAAAGATA TTGG-3′) and HCO2198 Rev.(5′ -TAA ACT TCW GGR TGW CCA AAR AATCA- 3′) (Cruaud et al., 2010). The specific primers were designed for *Nosema ceranae* according to (Chen et al., 2008) and the specificity were confirmed in ncbi-blast® software https://blast.ncbi.nlm.nih.gov. The forward primer (GGCAGTTATGGGAAGTAACA) and reverse primer (GGTCGTCACATTTCATCTCT).

The conventional PCR for COI and *Vairimorpha* (*Nosema*) *ceranae* amplification

The polymerase chain reactions were accomplished by EmeraldAmp Max PCR Master Mix (Takara, Japan, cat no. M711A) using Proflex thermal cycler (Applied Biosystems). *Nosema ceranae* product was detected with PCR program as follows: denaturation step at 95 ℃ for 5 min, then 35 cycles were run as follows: Secondary denaturation at 94 ℃ for 30 sec., annealing 55 ℃ for 45 sec., and extension at 72 ℃ for 1 min., then final extension step at 72 ℃ for 10 min. The PCR products were visualized on 1.5% agarose gel electrophoresis.

Sequencing of purified product

The amplified PCR products with the appropriate size were subsequently purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany, cat no. 28704). The purified PCR products were subjected to sequencing reactions using a Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, cat no. 4337455) according to the manufacturer's, the reaction products were purified by exclusion chromatography in DyeEX 2.0 Spin Kit (QIAGEN, Gmbh, German, cat no. 63204). The recovered materials were sequenced using a 3500XL Genetic (DNA) Analyzer (Applied Biosystems, Germany). A BLAST (Basic Local Alignment Search Tool) according to Abd El-Samie et al. (2021).

Quantitative PCR for *Nosema ceranae*

Sample preparation and DNA extraction

The studied samples for detection number of copies of *Vairimorpha* (*Nosema*) *ceranae* by using qPCR from the same studied groups (G1, G2, G4 and G7) were from raising virgins queens inside these colonies then dissect six new hatching virgin queens to get their ovaries

and Midguts (NvQO and NvQMg, respectively) and dissect too six new hatching workers to get their hypopharngeal glands and midguts (NWHg and NWMg respectively).

Six whole body of adult workers honeybees were homogenized from group7 (G7) and DNA was extracted from them and from the dissected specimens were performed using QI Aamp mini elute spin kit (Qiagen, GmnH, Germany, cat no. 51304).

RT-PCR were accomplished in triplicate using Quantifast SYBER Green PCR 2x master mix kit (Qiagen, Germany, cat no. 1044152) using specific primers of *Vairimorpha* (*Nosema*) *ceranae* and incubated in Proflex thermal cycler (Applied Biosystems) at denaturation step at 95 ℃ for 3 min, then followed by 40 cycles denaturation at 95 ℃ for 15 sec., annealing 60 ℃ for 10 sec., and extension at 72 ℃ for 10 sec. Then followed PCR amplification, melting curve analysis has been performed.

Standard Curve

Standard curve was generated with DNA amplicons with Nosema specific primers from qPCR performed with high positive samples for *Nosema ceranae* from infected honeybee group7 (G7) as a standard sample and used honeybee whole body (Wb). The amplicon plots of standards of Nosema concentration obtained using serial 10-fold dilutions of these samples were made with initial amount of the DNA amplicon obtained from a previous real-time RT-PCR and later purification. The next step was to quantify the product by spectrophotometer in Nanodrop and deduce the number of copies from the molecular weight of the DNA. The serial dilutions of the DNA from the group 7 (G7) as a standard sample from 18.8×10^6 copies to 188.0 copies. The DNA amplicons from dissected organs of new hatching virgin queen ovary (NvQO), virgin queen midgut (NvQMg), worker's hypopharengeal gland (NWHg) and worker's midgut (NWMg) from (C1, C2 and C4) were used as unknown samples.

Results

Microscopic detection

It was a proactive step from the beginning of developing an examination protocol, using light microscopy (ML 2000, Japan) as a standard method for initial examination the presence of *Vairimorpha* (*Nosema*) *ceranae* (*V. ceranae*) infection in all samples and *V. ceranae* were counted by hemocytometer slide (Paul Marienfeld GmbH, Germany). The results provided existence of *V. ceranae* in all marking colonies with different infection levels (Table1) and Figure1.

Table (1): Mean no. of *Vairimorpha ceranae* spores in bee of different honeybee colonies with different levels of *Vairimorpha ceranae* disease

Samples code	Mean No. of *Vairimorpha ceranae* Spores in Bee Worker ($\times10^6$)
C 1	0.90 ± 0.05^{d}
C 2	11.80 ± 0.10^{b}
C 4	10.45 ± 0.25^{c}
C 7	18.00 ± 0.10^{a}

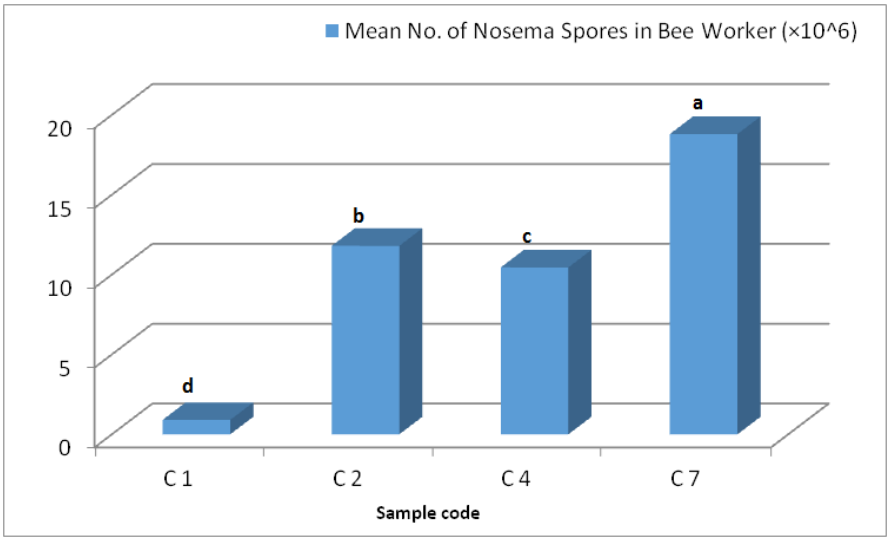

Figure (1): The means of *Vairimorpha (Nosema) ceranae* spores in bee workers in different colonies

DNA amplification

The product of PCR amplified fragments (690 bp) of COI gene (Figure 2a) and the phylogenetic tree showed the relationship result between COI and other sequence in GenBank showed 99% related to *Apis mellifera* and *Apis mellifera* haplotype Arabia with accession numbers OQ699249.1 and MT745906.1, respectively; and 78% similar to *Apis mellifera scutellata* and *Apis mellifera capensis* with accession numbers MG552698.1 and MG552695.1, respectively (Figure 2b & Supplemental material SF.2). The nucleotides differentiation revealed multiple cytosine-thymine (thymine–cytosine) transitions, an adenine-guanine (guanine-adenine) substitution and adenine-thymine (thymine-adenine) substitution Table (2) and (supplemental material SF.2).

The amplified fragment of *Vairimorpha (Nosema) ceranae* was 220bp from infected colonies in group 7 (G7). The sequence of *Vairimorpha (Nosema) ceranae* from C7 was recovered (Supplemental material SF.3), sequenced and was assigned in GenBank (https://ncbi.nlm.nih.gov/genbank/flatfile_changes/) with accession number OR793892 (Figure 3a). The results were similar to other species that were recorded in GenBank with 99.43% for *Vairimorpha* (*Nosema*) *ceranae* small subunit ribosomal RNA gene, partial sequence in accession number MN990268. and introduced with Neighbor-Joining tree (Figure 3b). The genetic distance value was found to be low (0.0-0.05) within the sequence of COI and *Vairimorpha (Nosema) ceranae* and others on GenBank.

Real time PCR

The quantity of target samples *N. ceranae* under study (new hatching virgin queen ovary (NvQO), virgin queen midgut (NvQMg), worker's hypopharengeal gland (NWHg) and worker's midgut (NWMg)) and the standard from forager honeybee whole body (Wb) from group 7 (G7) were subjected using qPCR by10-fold serial dilutions from 18.8×10^6 copies to 188.0 copies. In the results, the quantities which obtained from qPCR for each organ were summed separately for G1, G2, and G4 (Figure 4 a & b). In general, the results showed the number of copies of *Vairimorpha* (*Nosema*) *ceranae* higher in new hatched studied samples than old standard samples from the colonies in group 7 (G7).

Table (2): Variable nucleotides sites in COI sequence and others in GenBank

Accession number	132	175	265	427	457	485	580	590	592
lcl|Query_3465733	G	T	A	T	C	T	A	T	A
OQ699249.1	G	T	A	T	C	T	A	T	A
OK355216.1	G	T	A	C	C	T	A	T	A
MT919700.1	G	T	A	T	C	T	A	T	A
MT919698.1	G	T	A	C	C	T	A	T	A
MT745906.1	G	T	A	T	C	T	A	T	A
KP887097.1	G	T	G	T	C	T	A	T	A
KJ396191.1	G	T	G	C	C	T	A	C	T
MT745907.1	G	T	G	C	C	T	A	T	A
KP887095.1	G	C	G	T	C	T	A	T	A
KP163643.1	A	T	G	T	C	T	A	T	A
MG552698.1	G	C	A	T	T	C	A	T	A
MG552695.1	G	C	A	T	T	C	A	T	A
MG552683.1	G	C	A	T	T	C	A	T	A
MF678581.1	G	C	A	T	T	C	A	T	A
PP118059.1	G	C	A	C	T	C	G	T	A
PP118055.1	G	C	A	C	T	C	G	T	A
GU802455.1	G	C	A	C	T	C	G	T	A

The average was taken from the sum of the separated organs and calculated statistically with 3 replicates. The highest average quantity of *Vairimorpha (Nosema) ceranae* was in new-hatching worker's hypopharengeal gland (NWHg) with 25.49×10^6 copies while the lowest quantity was in G7 whole body (Wb) sample 18.8×10^6 copies which used as a standard and this quantity was very close to the quantity of new-hatching worker's midgut (NWMg) 19.89×10^6 copies. However, the quantities of *Nosema ceranae* in virgin queen (NvQO) and (NvQMg) were considered equal but these quantities were also higher than C7 sample and NWMg, on the other hand were lower than NWHg with differences were statistically significant ($P\leq 0.01$) Figure 5 and Table 3.

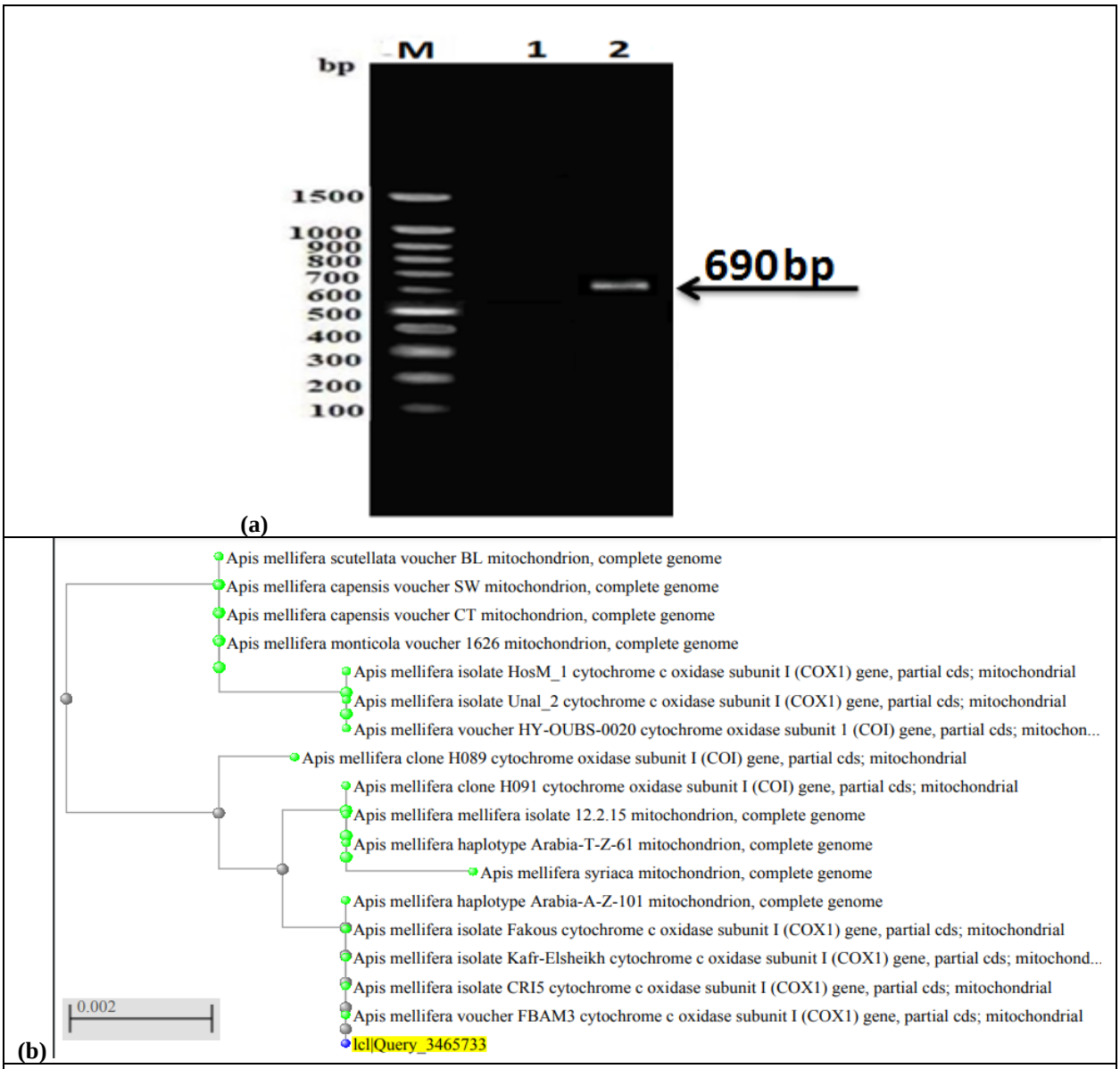

Figure (2): (a) Ethidium bromide-stained agarose gel (1.5%) electrophoresis of PCR amplified fragment for COI. Lane M: Molecular size marker (GeneDireX®), Lane 1: Negative control and Lane 2: 690bp COI fragment.
(b) Neighbor joining tree of CO1 sequence from *Apis mellifera* samples at GenBank site. https://www.ncbi.nlm.nih.gov/

Discussion

Cytochrome oxidase I analysis is a good marker for genetic differentiation between *A. mellifera* subspecies and its ecotypes (Moritz et al., 2005). Jones et al. (2004) and Abd El-Samie et al. (2021)'s results are consistent with our results which pointed to nucleotides substitutions and transitions cytosine-thymine (thymine–cytosine) transitions, an adenine-guanine (guanine-adenine) substitution and adenine-thymine (thymine- adenine) substitution in some sites comparable to other sequences in GenBank. At present, bee colonies are moved in search of pasture and different honey flow seasons, which increases the possibility of hybridization between bee strains. (De la Rua et al., 2009). Moreover, our result of mtDNA COI homology between 99%

and 78% for *Apis mellifera* haplotype of Arabia and *Apis mellifera capensis*, respectively, and also homolog with other subspecies in GenBank with varied identification percentage and nucleotide differentiation. This means that different subspecies of honeybee *Apis mellifera* are being inoculated, reducing the genetic markers of each subspecies or strain in each country, increasing the pathogens, diseases and possibly the introduction of other pathogens as mentioned before (Abd El-Samie et al., 2021). *N. Cerana* infecting a large scale of bee species included, European honeybees, Asian honeybees, and bumble bees (Plischuk et al., 2009; Chaimanee et al., 2010 & 2011). The wide host range of this parasite is of significant epidemiological concern (Chaimanee et al., 2011). *Nosema ceranae* was found in different major organs and tissues from studied honeybees and this pathogen is an important cause of colony death suddenly. Hemocytometer under magnification were used for spores counting as a first step of Diagnosis of *Nosema* spp. (Cantwell 1970). Therefore, the initial examination of *Vairimorpha* (*Nosema*) *ceranae* with a light microscopy was relied and using Hemocytometer counting slide. It was found that there was a noticeable difference in the number of spores between the colonies. After that, *V. ceranae* was diagnosed by RT-PCR with specific primers for small subunit ribosomal RNA (16s rRNA) and the amplified product was sequenced and phylogeny analysis showed the relation with others in GenBank (https://www.ncbi.nlm.nih.gov). Since 16S rRNA genes contain regions that are highly conserved among all organisms. This attribute rRNAs make them the most widely used molecular genetic marker Microbial studies (Chaimanee et al., 2011). It was interesting to know the reason for the difference of spores' amounts in the colonies, and it may be resulting from the difference in the spread of *Nosema ceranae* in the organs and tissues within the honeybees, and to detect this, it was necessary to use quantitative qRT-PCR. In previous studies, the main organs in honeybees were dissected to detect *Nosema ceranae*, and they indeed found it in these organs such as: ovaries and spermatheca of queens (Traver et al., 2012; Roberts et al., 2015), midgut and hypopharengeal glands (Jack et al., 2016; Li et al., 2019). Quantitative RT-PCR results illustrated the number of copies of *Nosema ceranae* in the ovaries of new hatching virgins' queens (NvQO) slightly more than in its midgut (NvQMg) in the same individuals with 22.99×10^6 and 22.62×10^6 copies, respectively. Our results were compatible with Traver et al. (2012) the infection levels of *N. ceranae* in older, mated queens were lower than newly emerged queens. While yolk undergoes degeneration in the ovaries, levels of vitellogenin (Vg), increase in the hemolymph of infected queens may be temporary condition due to deterioration of specific receptors on ovaries that facilitate uptake (Goblirsch, 2018). Therefore, ovaries had higher level of pathogen infection than the other tissues (Alaux et al., 2011; Traver et al., 2012) the vertical transmission is also possible as reported by Marín-García et al. (2022). Besides, *Nosema ceranae* could be transmitted horizontally from infected workers to the queen (Higes et al., 2009). Although the transmission of *Nosema ceranae* between the epithelial layers and the lumen of the midgut of adult infected honeybees (Goblirsch, 2018; Blot et al., 2023), in the current study, there were more copies of *Nosema ceranae* in the newly hatched virgin's queen midgut than newly hatched worker's midgut with means 22.62×10^6 and 19.89×10^6 copies, respectively. The high level of *Nosema ceranae* infection in queens that were imported and not native as (Muñoz et al., 2014) pointed out. On the other hand, qPCR results confirmed that newly hatched workers have a wide range number of copies of *Nosema ceranae* in their organs.

Since, the *Nosema ceranae* in the hypopharyngeal glands (NWHg) multiply higher than midguts (NWMg) at the same individuals with a significant means 25.49×10^6 and 19.89×10^6 copies, respectively. Hypopharyngeal glands were the most sensitive organ to infect with *V. ceranae* compared to other organs under investigation from newly hatched workers or newly hatched queens. Hypopharyngeal glands (HPGs) are very important in immune response and royal jelly production and HPGs secretion royal jelly proteins and glucosidase III (Vidau et al., 2014) and its total protein contents were significantly lower in *Nosema ceranae* infected bees (Li et al., 2019). In addition to, the infected hypopharyngeal glands in infected bees with *V. ceranae* suffering from atrophy, which leads nurse bees lose the ability of secretion royal jelly (RJ) and if the royal jelly produced will be contaminated with Nosema spores which reflected on decreased of production of mature larvae , young infected nurse bees cease brood rearing and turn to guarding and foraging duties, infected queens cease egg-laying, life expectancy is reduced as stated in previous studies (Higes et al., 2007; Goblirsch et al., 2013; Jack et al. 2016) and ends by colony collapse. The spore loads within the glands are altered by the hive infection levels and that disease transmission via these glands may be possible and may differ depending on colony infection levels and the periodic treatments operations against that pathogen, because presence of *Nosema ceranae* in these glands greatly suggests that food production and the building of comb can act as sinks and sources for *Nosema ceranae* and may increase the likelihood of disease dispersal within the hive (Copley and Jabaji, 2012), potentially making these tissues a new infection reservoir for *Nosema ceranae* (Traver and Fell, 2012). Therefore, the copies of the pathogen *Nosema ceranae* in new hatching of studied samples are more than old workers in the same colonies as shown in the results. *Nosema ceranae* can cause energetic stress in infected bees, resulting in gene expression changes associated with physiological homeostasis (Li et al., 2019). Finally, the using of qPCR helped to detect how many copies of *Nosema ceranae* in each studied organ from new hatched samples (and was found in other organs before in previous studies and mentioned above in the text) so the results have been suggested the pathogen could be existed in new generation of honey bee if the necessary measures are not taken to protect honey bees from a dangerous pathogen through permanent treatment and follow-up. Additionally, the results may indicate how this pathogen dispersal via vertical and horizontal transmission and this hypothesis because it is not clear till now also consistent with (Copley and Jabaji, 2012; Goblirsch, 2018; Marín-García et al., 2022). Therefore, it is necessary to enact the necessary laws to preserve local honeybee breeds and create isolated areas for their breeding. It is also good to tighten control over airports and sea, land and air transportation. To reduce smuggling between countries it is expected that these queens may not be subject to the programs used to combat bee diseases and become one of the main causes of disease transmission between countries.

Conclusion

The new hatching of virgin queen carried the Nosema spores in its ovaries could indicate the vertical transmission of the disease. Also, the presence of Nosema spores in other tissues as hypopharengeal glands and midguts of both new hatching individuals, the workers and virgin queen could indicate the horizontal transmission of disease. Both transmissions lead to sudden collapse or death in the colony. Due to the increased level of infection in the new generation and if no treatments or supplements improve the health of honeybees, the number of honeybees will gradually decline. So, these results demonstrate the importance of maintain proper quarantine

measures to reduce infection. In future studies, we hope to know which stage of the life cycle of *Vairimorpha* or *Nosema* from meronts to sporonts or sporoblast can be easily eliminated before it becomes a mature spore.

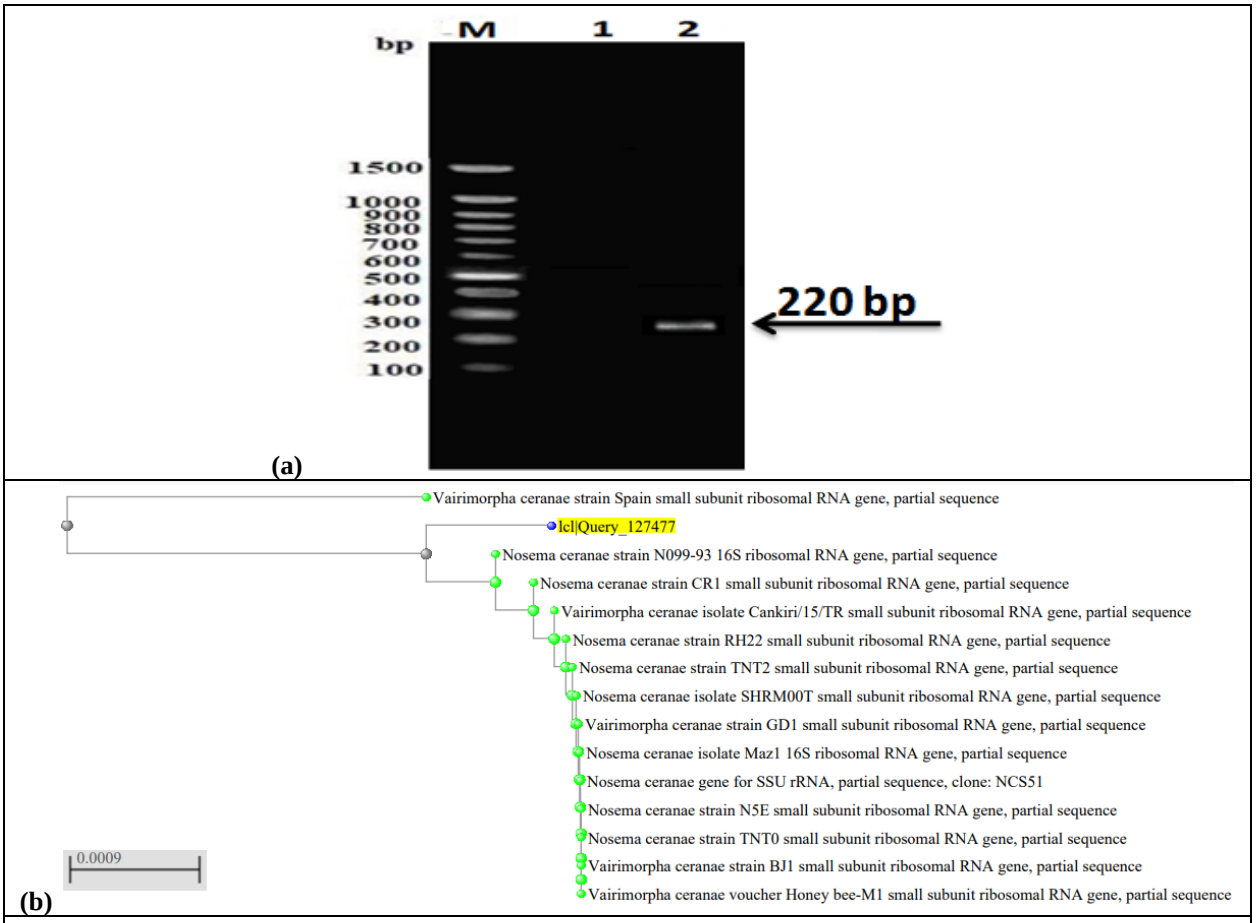

Figure (3): (a) Ethidium bromide-stained agarose gel of PCR amplified fragments. Lane M: Molecular size marker (GeneDireX®), lane1: Negative control and lane2: represent *Nosema ceranae* from the abdomen of forager bee samples. (b) Phylogenetic tree of the *Vairimorpha (Nosema) ceranae* based on 16S ribosomal RNA gene, partial sequence. The tree was constructed with the Neighbor-Joining tree.

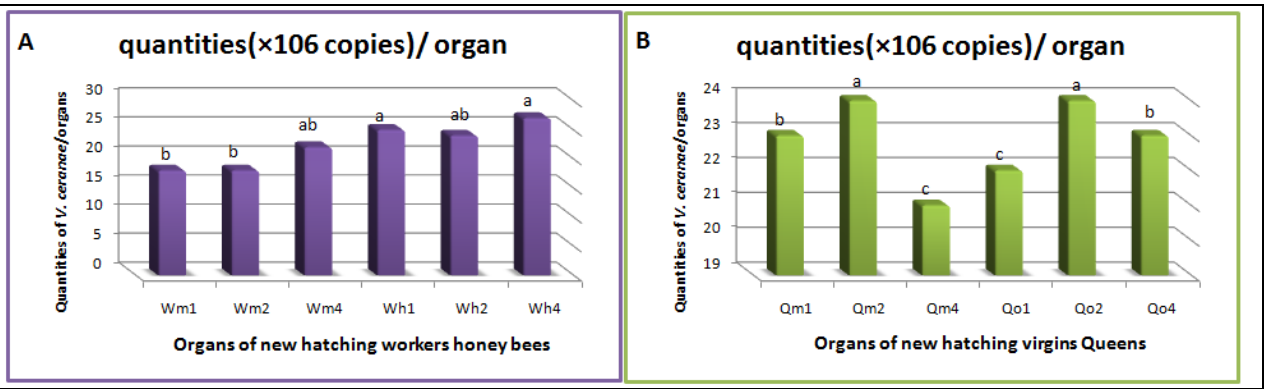

Figure (4): Showing the quantities of *Vairimorpha (Nosema) ceranae* in each organ in marking colonies (C1, C2 & C4); A) of new hatching workers honeybees (B) of new hatching virgin queen. Wm: worker's midgut, Wh: worker's hypopharengeal glands, Qm: virgin Queen's midgut and Qo: virgin Queen's ovary.

Table (3): Comparison of the most sensitive organ between queens and workers of newly hatched

Sample codes	Average of Quant. (×10^6 copies) ±SE
NWHg	**25.49±0.79^{a}**
NWMg	**19.89±1.52^{b}**
NvQMg	**22.62±0.93^{ab}**
NvQO	**22.99±0.44^{ab}**
Wb	**18.80±1.39^{b}**

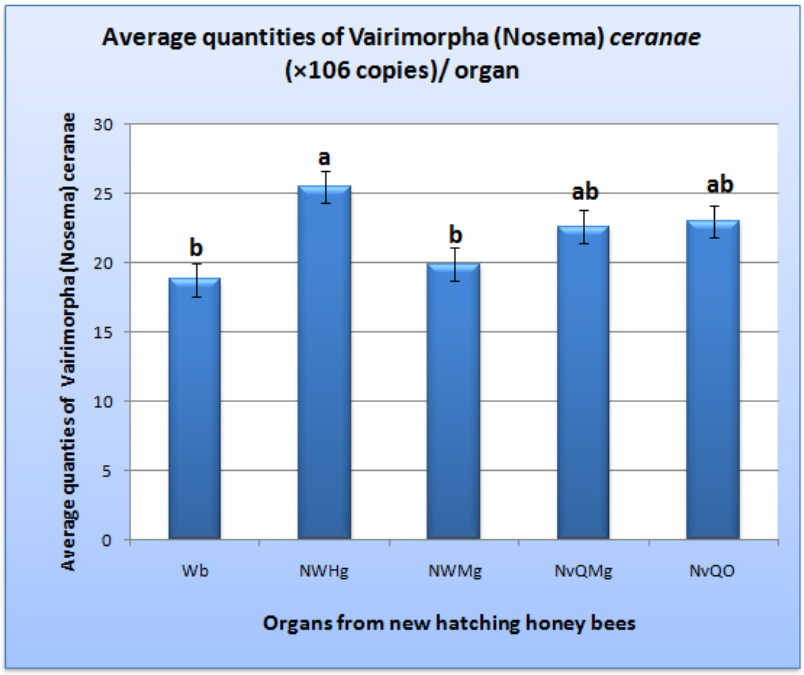

Figure (5): Comparison of the most sensitive organ between virgins queens and workers of newly hatched

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