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Research Article
Factors Contributing to Colony Collapse Disorder (CCD) in Iranian Honey Bee Colonies

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Abstract

Background and Objective: Honey bees are vital for agriculture, food security, medicine, and livelihoods, yet colony collapse disorder (CCD) increasingly threatens apiculture worldwide and in Iran. This study aimed to identify and analyze the parasitic, viral, bacterial, fungal and environmental contributors to CCD in Iran, with a focus on understanding disease patterns and seasonal correlations. **Materials and Methods:** From summer 2024 to spring 2025, forty apiaries with a history of CCD were sampled. Pathogens were screened through microscopic, molecular and culture-based techniques and data were statistically analyzed using SPSS. The investigation included identification of *Nosema* species, viral detection, fungal isolation and correlation testing between pathogen prevalence and CCD incidence across seasons. All data were analyzed using SPSS software, applying Chi-square, Fisher's exact and non-classical tests, with statistical significance set at $p<0.05$. **Results:** All *Nosema* infections were confirmed as *Nosema ceranae*. Among the five viruses screened, Deformed Wing Virus (DWV), Acute Bee Paralysis Virus (ABPV) and Chronic Bee Paralysis Virus (CBPV) were detected, with DWV reported for the first time in Iran. Seasonal fungal prevalence included *Penicillium* species in autumn and winter and Candida tropicalis was consistently present, linked to local humidity. A highly significant correlation was observed between *Nosema* infection and CCD during winter and spring ($p<0.001$), while viral infections showed a significant correlation with CCD in spring ($p<0.035$). Pathological examination confirmed characteristic *Nosema* lesions in bee intestines. **Conclusion:** This study highlights *Nosema ceranae* as the most critical contributor to CCD in Iranian honey bees, with viral and fungal pathogens playing seasonal roles. The findings provide a foundation for targeted disease management strategies, improved beekeeping practices and conservation of honey bee populations in Iran. Future studies should explore long-term monitoring, pathogen interactions and region-specific management approaches.

Key words: Honeybee, *Apis mellifera*, CCD, *Nosema ceranae*, *Deformed wing virus*, Iran

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Honey bees (*Apis mellifera*) are profoundly significant in modern human life, especially for Iranians, given their vital roles in agriculture, food and medicine production, fostering sustainable employment and their cultural importance. Their agricultural contribution is inherently tied to pollination. Individual foraging bees typically display "flower constancy", tending to visit flowers predominantly of a single plant species during a foraging trip[1]. This specific behavior makes honey bees essential pollinators. Moreover, with the increasing use of pesticides and notable human-induced environmental alterations, numerous natural pollinators are declining. However, honey bees, being manageable and mobile, can be extensively bred and deployed, effectively bridging the gap left by the depletion of other natural pollinators[2]. The significance of this insect is further highlighted by its seven main products: honey, honeydew, pollen, propolis, wax, royal jelly and venom. All these products possess considerable nutritional and medicinal attributes. Modern medicine increasingly uses them to treat various ailments, including gastrointestinal disorders. Propolis, for instance, is a complex compound known for its particular therapeutic effects, with its antibiotic properties being a defining characteristic[3]. Royal jelly is another bee product attracting attention as a potent tonic supplement and an ingredient in pharmaceutical formulations and cosmetic creams/lotions. Furthermore, honey's therapeutic benefits in addressing conditions like anemia and diverse digestive disorders are now firmly established[4]. Despite their immense benefits, honey bees, like other living organisms, are vulnerable to various pathogens and pests. These include *Varroa destructor, Acarapis woodi* and a range of fungal, bacterial and viral agents[5]. Among these threats, *Nosema* disease, caused by the microsporidian parasites *Nosema apis* and *Nosema ceranae*, represents a major concern. These obligate intracellular parasites invade the gut cells of adult honey bees, leading to impaired digestion, dysentery, a shortened lifespan and a general weakening of the colony[6]. While *N. apis* has traditionally been linked to temperate zones, *N. ceranae* has globally emerged as a more aggressive and prevalent pathogen. It often causes subtle, sub-clinical infections that contribute to colony decline without obvious symptoms[7,8]. These collective infections lead to the death of bees, reduced hive populations and economic losses.

In recent years, the widespread phenomenon of colony collapse disorder (CCD) has presented significant difficulties for beekeepers worldwide, including in Iran[9]. Numerous research teams are actively investigating the underlying cause(s) of this alarming population decrease. Since multiple sources identify specific parasitic diseases, especially *Nosema*, as either causative agents or contributing factors in this syndrome[5,7,9] a comprehensive examination of honey bee parasitic diseases and their connection to CCD is crucial. Prompted by a history of recent bee deaths in various areas of Iran and repeated concerns from beekeepers regarding the severity of these losses, we initiated a study. The study objective was to conduct periodic sampling to evaluate the contamination status of apiaries across different seasons. The primary goals were to pinpoint the main causes of mortality and to investigate the correlation between parasitic infections (especially *Nosema*) and the CCD syndrome in the region's apiaries. By identifying these key causative agents, we hope to contribute to controlling these losses and providing beekeepers with preventative strategies. Therefore, in addition to examining parasitic infections, this research also explored the impact of various viral, bacterial and fungal factors, as well as climatic conditions, environmental humidity and apiary management practices.

MATERIALS AND METHODS

Study design: This study adopted a preliminary longitudinal (prospective) design to investigate the correlation between the incidence of parasitic infections and colony losses in apiaries with a history of decline. The apiary was considered the primary sampling unit.

Sample size and sampling procedure: A total of 40 apiaries were selected, ensuring proportional representation based on the number of apiaries in each section. primarily targeted apiaries that had experienced colony collapse disorder (CCD) in previous years. To ensure broad environmental representation, samples were collected from all three prevailing climatic zones: Mountainous, tropical and temperate.

Sampling occurred throughout each of the four seasons, starting in summer 2024 and concluding in spring 2025. The 40 selected apiaries were initially categorized into "loss" and "no-loss" groups based on operational definitions of summer colony loss. This categorization and subsequent sampling for parasitic and other infections were started in the summer and repeated for the same 40 apiaries in autumn, winter and spring.

From each selected apiary, 10% of the hives were chosen for sampling, in accordance with guidelines from the national veterinary organization. Bees from each selected hive were transferred into sealed glass containers, gently shaken to

anesthetize them, then moved to freezer bags and transported to the Veterinary Laboratory for initial assessment. Macroscopic and microscopic examinations were performed to identify the presence of *Varroa*, *Nosema*, Acarapis and Protozoa (Gregarines and *Malpighamoeba mellificae*).

Laboratory diagnosis:

***Nosema* infection quantification:** To quantify *Nosema* infection, the abdomens of 25 bees were isolated. These were homogenized in 2 mL of distilled water using a porcelain mortar. A drop of the resulting suspension was then placed on a hemocytometer, covered with a coverslip and observed under a microscope at 40x objective. *Nosema* spores appeared as transparent, oval bodies (4-6 µm×2-4 µm). Infection intensity was determined by the number of *Nosema* spores per bee using the following formula[10]:

$$\text{Number of spores per bee} = \frac{\text{Average number of spores counted} \times \text{Dilution factor} \times \text{Hemocytometer factor}}{\text{Number of bees sampled}}$$

Gregarine infection detection: Gregarine infection was assessed following the method described by Bessette *et al*.[11]. Abdomens from 25 bees were homogenized and wet mounts were prepared from the suspension for microscopic examination at 40x objective.

***Malpighamoeba mellificae* detection:** *Malpighamoeba mellificae* cysts (5-8 µm) were identified by microscopic examination of the Malpighian tubules. The entire digestive tract and Malpighian tubules were extracted from adult bees using fine-tipped forceps. After separating the Malpighian tubules, they were crushed on a slide under a coverslip and examined at a 40x objective[12].

***Varroa destructor* infestation quantification:** *Varroa destructor* infestation was determined using the method described by Colin *et al*.[13]. At least one hundred bees were placed in a glass container with hot water and a drop of detergent (to aid mite detachment). After vigorous shaking, the liquid was poured through a double-layered filter, separating bees (upper layer) from mites (lower layer). The percentage of infestation was then calculated.

***Acarapis woodi* detection:** *Acarapis woodi* detection followed Colin's method[13]. Discs of thoracic segments (between the first and second leg pairs) were excised using a scalpel. These discs were digested in 10% KOH at 60°C for 30 min to clear tissues and trachea. Prepared sections of tracheae were then examined under a light microscope at 10x objective for the presence of mites. For further specialized analyses, samples were stored at -20°C and transported with ice to the Faculty of Veterinary Medicine, University of Tehran.

Fungi detection: A sample from the mummified larvae or hive debris was cultured in the lab for a definitive diagnosis and species identification. The sample was placed on a nutrient medium, such as Sabouraud Dextrose Agar (SDA) and was incubated at a specific temperature (usually 25-30°C). Fungal colonies grew over several days and these were then examined microscopically for their morphology and biochemical tests were performed to confirm the species.

Supplementary analyses:

Pathological examination of *Nosema*-infected bee intestines: To assess the pathological effects of *Nosema* on honey bee intestines, the method by Dussaubat *et al*.[14] was used. The digestive tracts of *Nosema*-infected bees were extracted with fine-tipped forceps and fixed in 10% formalin for 24 hrs. Fixed samples were then embedded in paraffin and 4 µm sections were prepared using a microtome. These sections were mounted on slides, stained with hematoxylin-eosin and examined at the Pathology Department, Faculty of Veterinary Medicine, University of Tehran.

***Nosema* species identification:** *Nosema* species were identified by DNA extraction from spores using a specialized MBST kit, followed by PCR amplification using species-specific primers targeting the 16S ribosomal RNA gene[15].

DNA extraction: Performed using the MBST (Molecular Biological System Transfer) kit according to the manufacturer's instructions.

PCR amplification: Utilized specific forward and reverse primers for *N. ceranae* (218 ceranae MITOC FOR/REV) and *N. apis* (321 apis FOR/REV) based on the 16S rRNA gene sequence. All steps were performed on ice and samples were run on a Primus 96 plus thermocycler (MWG). A negative control (distilled water instead of the sample) was included in both the extraction and PCR stages. Amplified DNA products were resolved on a 1.5% agarose gel (Sinagen). Gel preparation involved dissolving agarose in TBE buffer, adding Safe Red stain and running at 100V for 45 min. Gels were visualized using an IN GENIUS transilluminator (Sinagen).

Viral infection assessment: Target apiaries were selected based on previous years' population decline symptoms. Viral RNA was extracted from bee bodies using specialized kits (CinnaPure RNA, Sinagen). Bee bodies were finely minced with a sterile scalpel and homogenized before RNA extraction. cDNA was synthesized from 5 µL of extracted RNA by adding 1 µL of Random Hexamer and incubating at 65°C for 5 min in an MJ Mini thermocycler (BIO RAD). RT-PCR was then performed u sing specific primers to detect five key honey bee viruses: ABPV, BQCV, CBPV, DWV and KBV. Due to the absence of positive controls, positive samples were sequenced and confirmed before inclusion in the results.

- **RNA extraction:** Performed using the CinnaPure RNA kit (Sinagen) following the manufacturer's instructions, involving mechanical disruption and homogenization of bee bodies
- **cDNA synthesis:** A 5 µL of extracted RNA was reverse-transcribed using Random Hexamer and incubated at 65°C for 5 min

PCR primers:

- **ABPV (RdRp gene, 452 bp):** F-5'-TGAGAACACCTGT AATGTGG-3', R-3'-ACCAGAGGGTTGACTGTGTG-5'
- **BQCV (Structural Polyprotein, 700 bp):** F-5'-TGGTCAGCT CCCACTACCTTAAAC-3', R-3'-GCAACAAGAAGAAACGTAAA CCAC-5'
- **CBPV (RNA Polymerase, 570 bp):** F-5'-TCAGACA CCGAATCTGATTATTG-3', R-3'-ACTACTAGAAACTCGTCG CTTCG-5'
- **DWV (Structural Polyprotein, 194 bp):** F-5'-CTTAC TCTGCCGTCGCCCA-3', R-3'-CCGTTAGGAACTCATTAT CGCG-5'
- **KBV (Non-structural Polyprotein, 415 bp):** F-5'-GATGAACGTCGACCTATTGA-3', R-3'-TGTGGGTTGGCTATG AGTCA-5'

A negative control (distilled water instead of sample) was included in both RNA extraction and PCR stages. Amplified DNA products were resolved on a 2% agarose gel (Sinagen) prepared with TBE buffer and Safe Red stain. Samples (8 µL of PCR product) and a marker were loaded and electrophoresis was conducted at 100V for 45 min. Gels were visualized using an IN GENIUS transilluminator (Sinagen).

Statistical analysis: All data obtained from this research were statistically analyzed using SPSS software. Both classical statistical methods, including Chi-square tests and Fisher's exact test and non-classical methods were employed for data analysis. A significance level of $p<0.05$ was considered for all statistical tests.

RESULTS

Demographic and apiary characteristics: The apiaries included in this study comprised 40 to 450 hives, with two notable exceptions having 800 and 1200 hives, respectively. Sampled hives typically contained 6 to 10 frames, averaging 8 frames per hive, which generally aligns with industrial beekeeping standards. Observations indicated that none of the selected apiaries had ventilation openings opposite the hive entrance and plastic bags were commonly used as hive covers. This study investigated various infections in 40 apiaries across different seasons. In the summer sampling period, only one apiary experienced colony loss. Among the 40 apiaries examined in summer, 19 were infected with *Nosema*. Molecular analysis using PCR, which specifically distinguishes *Nosema apis* from *Nosema ceranae*, revealed that all *Nosema* infections were exclusively caused by *N. ceranae*. Characteristic amplification bands of 218 bp for *N. ceranae* were observed on agarose gels, whereas no bands corresponding to *N. apis* were detected (Fig. 1 and 2). Additionally, 22 apiaries (55%) were infested with *Varroa destructor* during the summer. Microscopic examination revealed low-level yeast contamination across all apiaries, consistent with the normal gut flora of healthy honey bees. No infections were detected for *Acarapis woodi*, *Malpighamoeba mellificae*, gregarines, or other fungal pathogens. To further investigate the lesions caused by *N. ceranae*, both the presence and intensity of infection were assessed, accompanied by detailed histopathological evaluation of midgut tissues. The major pathological features are presented in Fig. 3-14. Figure 3 shows the early-stage pathological changes in the midgut epithelium, including mild vacuolization and localized disruption of epithelial cell boundaries. Figure 4 illustrates the progressive degeneration of epithelial cells and partial destruction of the peritrophic membrane. Figure 5 presents extensive vacuolization and necrosis within the epithelial layer, indicating severe tissue damage in heavily infected bees. Figure 6 demonstrates complete disintegration of the peritrophic membrane and loss of cellular integrity. Figure 7 depicts the accumulation of *Nosema ceranae* spores within epithelial cells.

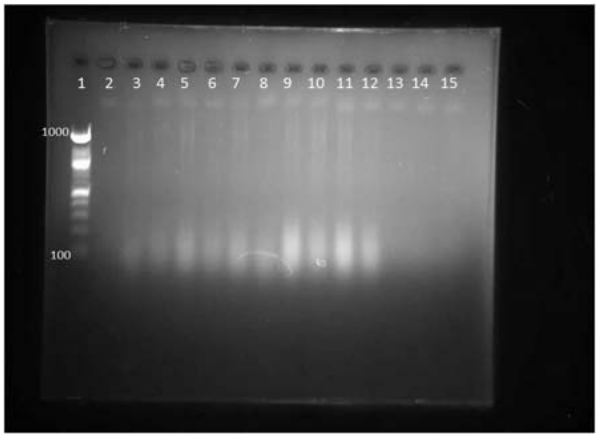

Fig. 1: Results from adult bee samples using *Nosema apis*-specific primers

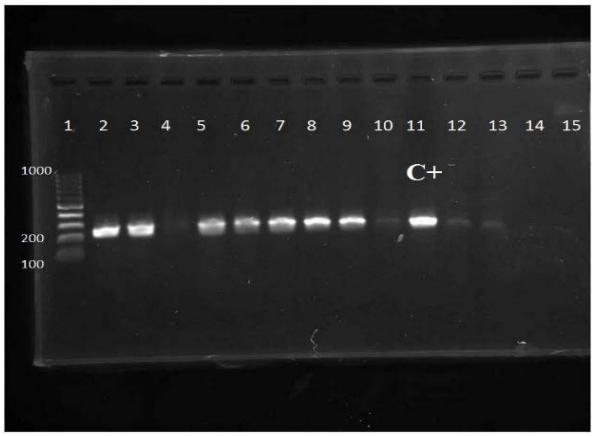

Fig. 2: Results of adult bee samples using *Nosema ceranae*-specific primers

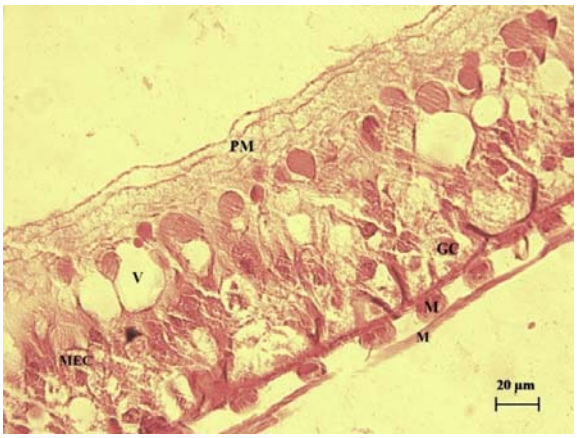

Fig. 3: Microscopic view of a normal honey bee midgut, showing the peritrophic membrane, epithelial cells and cross-sections of both circular and longitudinal midgut muscles

M: Muscle, GC: Generator cell, PM: Peritrophic Membrane, V: Vacuole, MEC: Mature epithelial cell, H&E staining and Scale: 20 µm

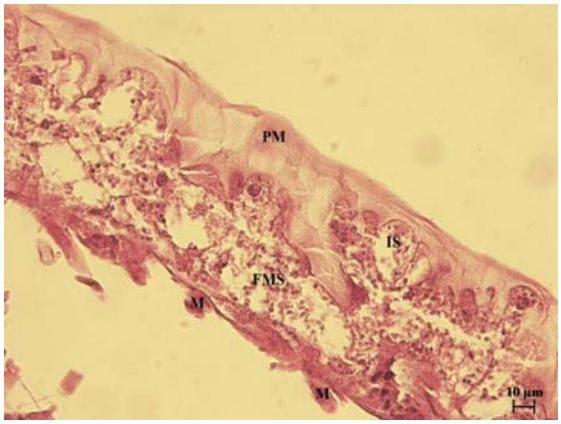

Fig. 4: *Nosema ceranae* infection of the midgut mucosa in an infected honey bee, both intracellular and extracellular forms of *Nosema ceranae* are visible

Peritrophic membrane remains intact, PM: Peritrophic membrane, M: Muscle, FMS: Free mature spores, IS: Intracellular immature spores, H&E staining and Scale: 20 µm

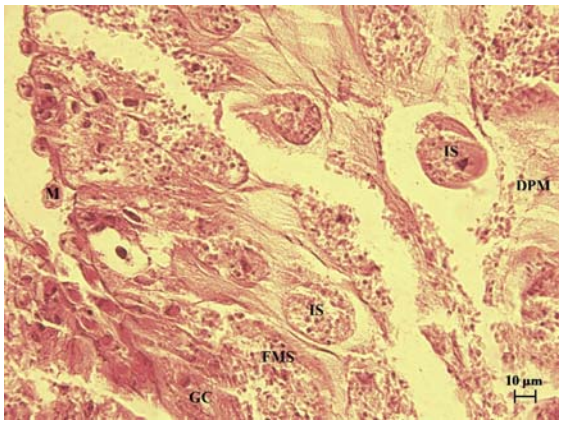

Fig. 5: Intracellular and extracellular *Nosema ceranae* organisms in the midgut mucosa, showing ruptured infected cells releasing microorganisms

M: Muscle, IS: Immature intracellular spores, FMS: Free mature spores, DPM: Disrupted peritrophic membrane, GC: Generating cell, H&E staining and Scale=10 µm

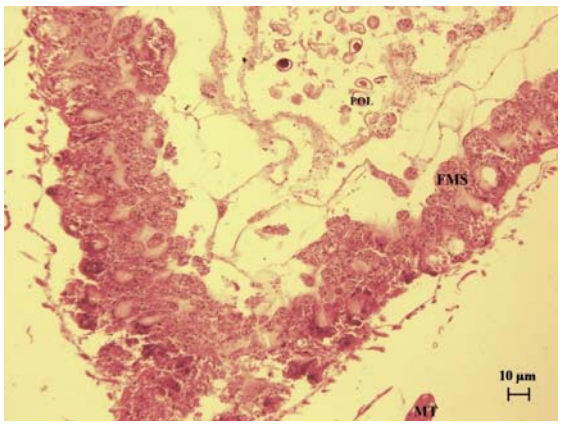

Fig. 6: Lower magnification showing widespread infection of the midgut mucosal tissue of a honey bee with *Nosema ceranae*

Over 90% of the mucosal tissue is destroyed, Plant pollen grains, remnants of the peritrophic membrane and numerous *Nosema* spores are visible within the intestinal lumen, POL: Pollen, FMS: Free mature spores, MT: Malpighian tube, H&E staining and Scale=10 µm

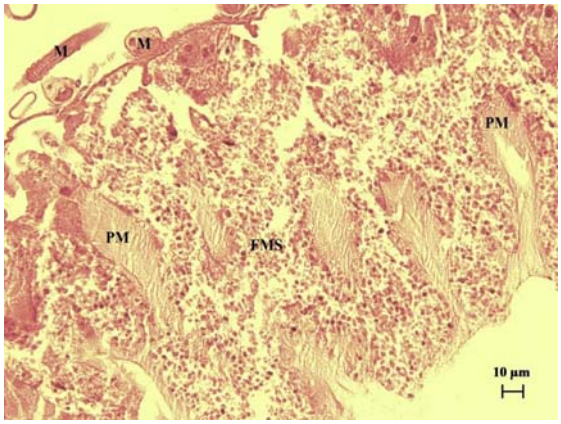

Fig. 7: Microscopic view of the complete destruction of midgut epithelial cells in a *Nosema ceranae*-infected bee, with replacement by mature *Nosema ceranae* spores
Remnants of the peritrophic membrane are evident, FMS: Free mature spores, PM: Peritrophic membrane, M: Muscle, H&E Staining and Scale: 10 µm

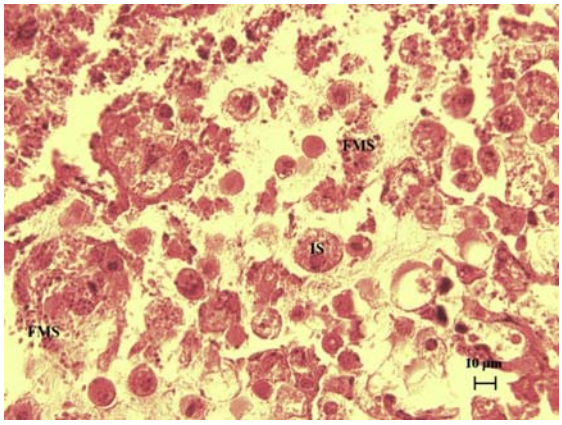

Fig. 8: Immature intracellular stages and free spores, along with peritrophic membrane remnants, in the midgut lumen of a honey bee infected with *Nosema ceranae*
FMS: Free mature spores, IS: Immature intracellular spores, H&E staining and Scale=10 µm

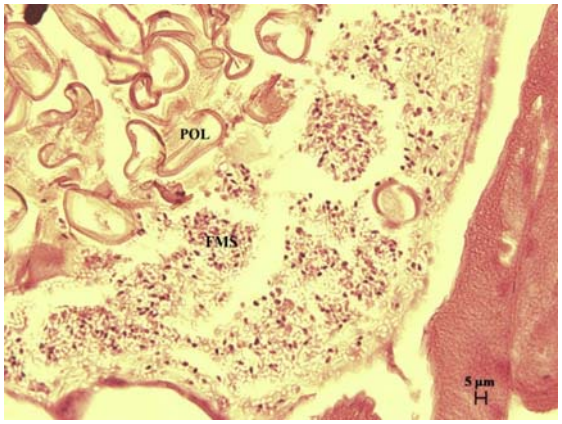

Fig. 9: Higher magnification showing *Nosema ceranae* free spores within the midgut lumen, alongside ingested pollen grains, tissue debris and peritrophic membrane remnants.
Pol: Pollen, FMS: Free mature spores, H&E staining and Scale: 5 µm

Figure 8 shows the presence of spores in the intestinal lumen and disruption of the mucous layer. Figure 9 highlights the advanced infection phase, where numerous spores aggregate in both epithelial and luminal regions. Figure 10 compares healthy and infected tissues, clearly showing epithelial rupture and luminal debris. Fungal and viral contamination in honey bee samples was also assessed. *Penicillium* (Fig. 11) and *Candida tropicalis* (Fig. 12) are shown with colony morphology and microscopic structures. Acute Bee Paralysis Virus (ABPV, Fig. 13) and Deformed Wing Virus (DWV, Fig. 14) were detected by PCR, illustrating infection levels across apiaries. No *Acarapis woodi* was observed.

Nosema ceranae infection in honey bees causes damage to various midgut epithelial structures, including the peritrophic membrane, mature epithelial cells and epithelial progenitor cells. The observed pathological changes were graded into five categories based on severity: 0 (no changes, normal midgut structure), 1 (mild infection, 10-30% affected), 2 (moderate infection, 30-50% affected), 3 (moderately severe infection, 50-70% affected) and 4 (severe infection, 70-100% affected). Microscopic analysis of *Nosema ceranae* infection in bee midguts revealed several key pathological changes, graded by severity. These included disruption of the peritrophic membrane, detachment and necrosis of epithelial cells and a general loss of histological structure, with the extent of extracellular and intracellular *Nosema* forms correlating with infection intensity. Infected cells appeared swollen, sometimes rupturing and releasing spores. In severe cases, over 90% of the intestinal mucosa was compromised and the midgut's structure was often destroyed, filled with free spores and cellular debris. Notably, no significant pathological changes were observed in the muscular layers or Malpighian tubules (Table 1).

In the autumn season, 4 apiaries (10%) experienced colony loss, while 26 apiaries (65%) were infected with *Nosema ceranae* and 19 apiaries (47.5%) were infested with *Varroa destructor*. No cases of *Acarapis woodi*, *Malpighamoeba mellificae*, or gregarine infections were observed during this season. Out of the 40 apiaries examined in autumn, 3 apiaries (7.5%) were infected with *Penicillium* fungus and this infection led to mortalities within those apiaries. The morphology of *Penicillium* fungus is illustrated in Fig. 11a-b.

During the winter season, 38 out of 40 apiaries (95%) experienced colony losses. Of the surveyed apiaries, 34 (85%) were infected with *Nosema ceranae*, while 5 apiaries (12.5%) were infested with *Varroa destructor*. No cases of *Acarapis woodi*, *Malpighamoeba mellificae*, or gregarine infections were observed in this season. Furthermore, 5 apiaries (12.5%) showed *Penicillium* mold contamination, with one of these

Table 1: Pathological findings and damage severity grading of *Nosema ceranae*-infected honey bee midguts (studied cases)

Study case code	Spore count (Smear)	Grading	Damage severity code	Complete mucosal tissue disorganization/loss	Epithelial cell sloughing into lumen	Intracellular immature spores	Extracellular spores	Peritrophic membrane disruption
1	-	4	≤90%	+	+	+	+	+
2	+	1	10-15%	-	-	+	+	-
3	++	3	65-70%	±	+	+	+	+
4	+++	4	≤90%	+	+	+	+	+

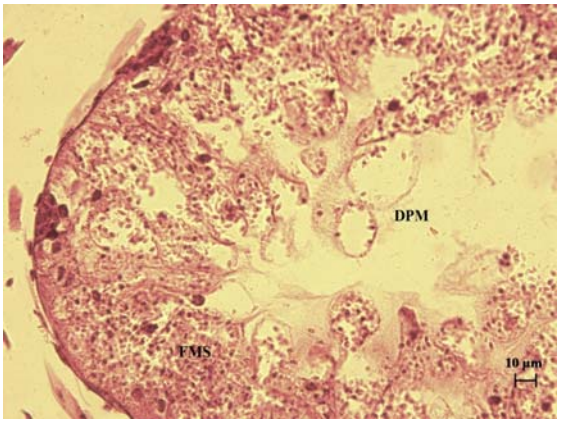

Fig. 10: Close-up view of severe midgut epithelial infection in a honey bee affected by *Nosema ceranae*
Over 90% of the midgut epithelium has been destroyed due to this infection, numerous *Nosema* bodies, appearing as oval shapes with polar filaments, are observed, DPM: Disrupted peritrophic membrane, FMS: Free mature spores, H&E staining and Scale: 10 µm

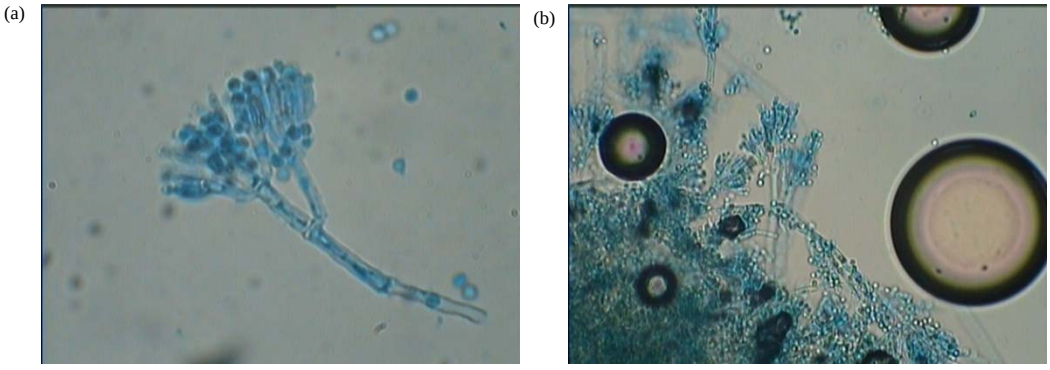

Fig. 11(a-b): *Penicillium* contamination of honey bee samples, (a) Macroscopic growth of characteristic colonies on sabouraud dextrose agar and (b) Microscopic examination showing hyphae and conidial structures, confirming fungal

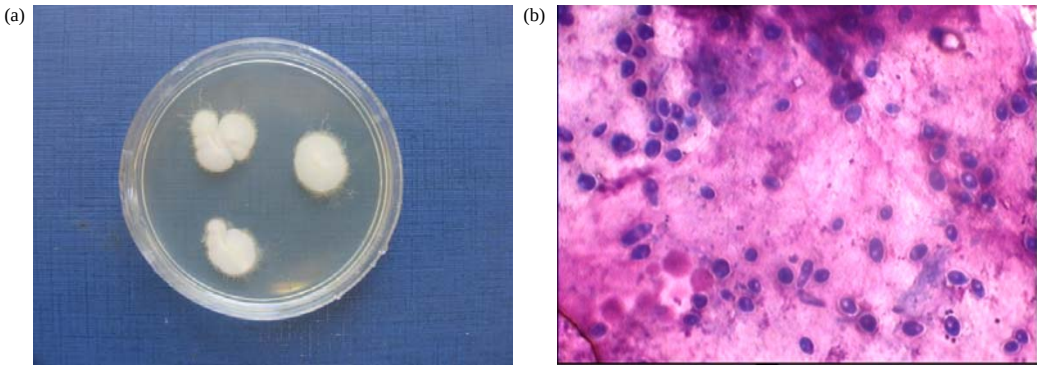

Fig. 12(a-b): Identification of *Candida tropicalis* infection in honey bee samples, (a) Macroscopic view of fungal growth on culture medium, showing colonies characteristic of *C. tropicalis* and (b) Microscopic examination confirms the presence of budding yeast cells and pseudohyphae typical of *C. tropicalis*

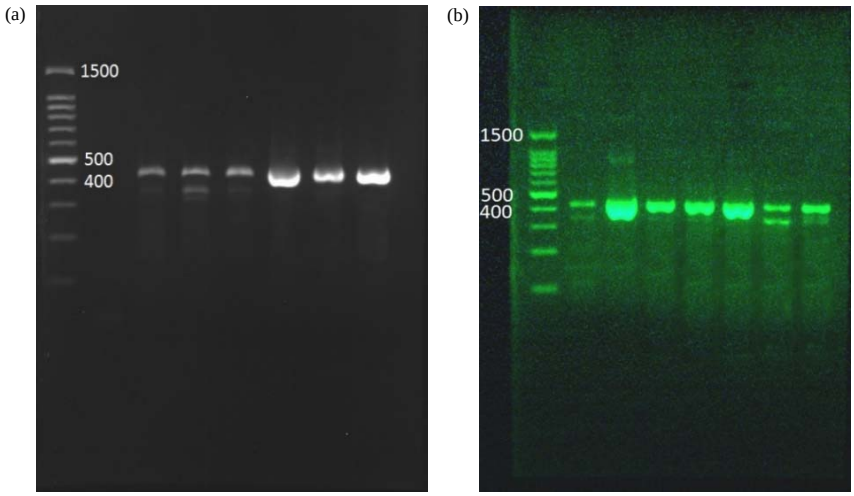

Fig. 13(a-b): Results of ABPV contamination in apiaries using specific primers, (a) PCR results for the detection of ABPV infection in apiary samples using specific primers, green bands indicate positive samples and reflect the infection intensity in each sample and (b) Comparison between different apiaries or sample sets, illustrating variations in ABPV infection levels

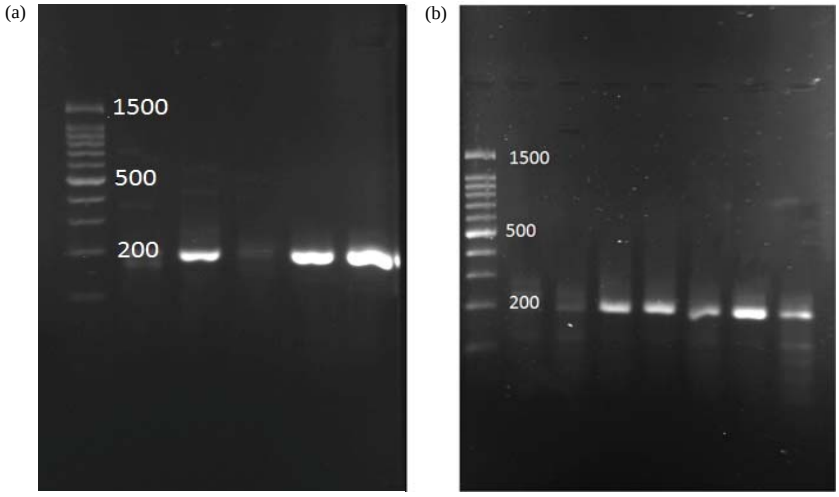

Fig. 14(a-b): Results about the investigation of apiary infestation with Deformed Wing Virus (DWV) utilizing specific primer sets, (a) PCR results for the detection of DWV using specific primers in bee samples. Positive samples are commonly associated with the presence of Varroa destructor and (b) Distribution pattern of DWV infection across different apiaries, illustrating variations in infection intensity among samples

Table 2: Comparison of results from apiary examinations across different seasons

			Infection													
	Colony loss		*Nosema*		*Varroa*		Yeast		Mold		CBPV		ABPV		DWV	
season	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Summer	1	2.5	19	47.5	22	55	40	100	0	0	Untested	-	Untested	-	Untested	-
Autumn	4	10	26	65	19	47.5	40	100	3	7.5	Untested	-	Untested	-	Untested	-
Winter	38	95	34	85	5	12.5	40	100	5	12.5	5	12.5	1	2.5	3	7.5
Spring	35	87.5	35	87.5	4	10	40	100	0	0	0	0	13	32.5	9	22.5
total	78		114		50		160	100	8		5		14		12	

apiaries exhibiting co-infection with both *Penicillium* mold and *Candida tropicalis* yeast. In total, 9 apiaries (22.5%) were identified with viral infections during winter. Specifically, 5 apiaries were infected with CBPV (Chronic Bee Paralysis Virus), 3 with DWV (Deformed Wing Virus) and 1 with ABPV (Acute Bee Paralysis Virus). Following the observation of yeast presence on some samples, these were submitted to the Mycology Laboratory at the Faculty of Veterinary Medicine, University of Tehran, for identification. Subsequent analyses confirmed the presence of *Candida tropicalis*, as illustrated in Fig. 12a-b.

In the spring, 35 apiaries (87.5%) experienced colony collapse and an equal number (87.5%) were found to be infected with *Nosema ceranae*. Additionally, 4 apiaries (10%) were infested with *Varroa destructor*. No infections with *Acarapis woodi*, *Malpighamoeba mellificae*, gregarines, or fungi were observed during this season. Viral screening conducted due to the high rate of colony losses revealed the presence of two viruses: Acute Bee Paralysis Virus (ABPV) and deformed wing virus (DWV). Figure 13a presents the PCR results for the detection of ABPV infection in apiary samples using specific primers. The green bands represent positive samples, indicating the intensity of infection in each case. Figure 13b compares different apiaries, highlighting the variation in ABPV infection levels across the study sites. Similarly, Fig. 14a shows the PCR amplification results for DWV detection in bee samples using specific primers. Positive samples were commonly associated with *Varroa destructor* presence. Fig. 14b depicts the distribution pattern of DWV infection among different apiaries, illustrating variations in infection intensity and demonstrating the relationship between viral load and colony collapse. A molecular analysis for BQCV and KBV was also conducted in this study, but no cases of infection with either virus were observed.

Table 2 presents a comparative analysis of beehive colony losses and their contamination with various parasitic, fungal and viral agents across different seasons. The highest colony losses were observed during winter and spring, with rates of

95% and 87.5%, respectively, while the lowest rates occurred in summer and autumn. Regarding parasitic infections, *Nosema* contamination was present in all seasons, with the highest prevalence in winter (85%) and spring (87.5%). This pattern contrasts with *Varroa* mite contamination, which was most prevalent in summer and autumn. Fungal contamination was consistently observed across all seasons, with yeast species, specifically *Candida tropicalis*, identified in 100% of the cases, although with low intensity. Mold contamination, specifically *Penicillium*, was recorded only in autumn (7.5%) and winter (12.5%). For viral contamination, due to high costs, samples were analyzed only in winter and spring. The Chronic Bee Paralysis Virus (CBPV) was detected exclusively in winter at a rate of 12.5%. Acute Bee Paralysis Virus (ABPV) and Deformed Wing Virus (DWV) were both found in winter and spring. ABPV infection rates were 2.5% in winter and 32.5% in spring, while DWV rates were 7.5% and 22.5% for the same seasons, respectively. This study investigated a range of common honey bee pests, including the *Meloe beetle*, *Braula coeca*, the Greater Wax Moth (*Galleria mellonella*), the Lesser Wax Moth (*Achroia grisella*) and myiasis-causing agents. None of these pests were observed in the apiaries under investigation.

DISCUSSION

Reports of significant honey bee colony losses in the U.S. began in late 2006. Due to the rapid and unusual nature of this decline, beekeeping and bee disease experts termed the phenomenon colony collapse disorder (CCD)[16]. Global reports from 2006 to 2008 indicated that beekeepers experienced widespread losses, with rates ranging from 31.8% to 35.8%. Despite extensive research efforts, the definitive cause or causes of CCD have not yet been conclusively identified[16]. The commercial pollination value of honeybees in the United States is estimated at $15-20 billion annually. Over recent years, colony collapse disorder (CCD) has caused a severe decline in bee populations, resulting in significant damage to the global beekeeping industry[17]. Colony collapse disorder (CCD) is defined by the sudden disappearance of adult worker bees, with no dead bees found in or around the hive. This is unusual because adult bees fail to return, leaving the colony to decline without sufficient care. The lack of dead bees makes it very difficult to identify the cause of the syndrome[16]. Reports from Iran documented widespread honey bee population declines, characterized by reduced worker bee numbers, a live queen and remaining brood with a lack of nurse bees.

In the current study, the most significant honey bee population decline (92.5% in winter, 85% in spring) coincided with the highest prevalence of *Nosema* infection (around 85%). This finding is consistent with other studies from Iran[18]. Historically, *Nosema apis* was the primary species, but molecular methods have since identified *Nosema ceranae* as a key pathogen[19]. Using PCR, this research confirmed that only *Nosema ceranae* was present in our bee colonies. These results are consistent with other Iranian studies, which indicate that *N. ceranae* is the predominant *Nosema* species in the region[20,21]. While some studies have shown a strong link between *Nosema* and colony collapse disorder[22,23], others have found no significant association[24]. This variation highlights the complex nature of CCD and the need for more research in different environmental contexts. Pathological examination of honey bee guts infected with *Nosema ceranae* revealed damage to multiple intestinal structures, including the peritrophic membrane, epithelial cells and regenerative cells. The severity of these lesions generally correlated with spore counts from wet mounts. However, in some cases where no spores were detected on wet mounts, histopathological analysis confirmed the infection. This discrepancy may be due to the peritrophic membrane remaining intact despite severe infection, preventing the release of spores. Such findings suggest that the severity of lesions might not always correlate with the presence of spores in feces. A similar conclusion was reached by Vidau *et al.*[25], who found that histopathology was more sensitive and specific than microscopic examination for detecting *Nosema*, even in asymptomatic colonies. This higher sensitivity supports a potential link between *Nosema* and colony collapse disorder. Dussabat *et al.*[14] further showed that the pathological changes in the honey bee midgut caused by *N. ceranae* are consistent across different climatic regions, indicating that the parasite's mechanism of damage to the gut is similar regardless of location.

Varroa destructor remains a major global problem for beekeeping. The current study found the highest infestation rates in late summer and autumn. This aligns with findings from Smoliński *et al.*[26] in Central Europe, who linked high autumn infestation to significant colony losses, which the low presence or absence of covered larvae cells could explain. However, the study found no significant association between *Varroa* infestation and bee loss ($p>0.05$).

No evidence of *Acarapis* mites or the protozoan *Malpighamoeba mellificae* was found in any of the studied colonies. This finding suggests that the mite *Acarapis woodi* is not widespread in Iran, a conclusion that is consistent with other local studies. However, this contrasts with reports from

Russia[27] and Korea[28], as well as previous reports of Iran, in which various *Acarapis* species have been detected. These differences in prevalence may be due to varying climatic conditions and the widespread use of anti-varroa treatments, which can also affect *Acarapis* mites.

In the present study, yeast contamination was observed in all seasons with low intensity and laboratory analysis identified the species as *Candida tropicalis* (100% prevalence). Mold contamination, identified as *Penicillium* spp., was detected in eight cases, with prevalence rates of 7.5% and 12.5% in autumn and winter, respectively. The higher prevalence in these seasons is attributed to increased humidity. Field observations revealed that the use of nylon sheets on hive tops and inadequate ventilation increased internal hive humidity, promoting fungal growth. The implementation of additional ventilation holes and the replacement of plastic sheets with cloth coverings successfully controlled the fungal contamination and subsequent losses. While other studies, such as Nabian *et al.*[29], have isolated various fungal species, including *Penicillium* and *Candida*, from honeybees, our statistical analysis found no significant correlation between fungal factors and colony collapse disorder ($p>0.05$).

Viral screenings, conducted on samples from the winter and spring seasons, revealed significant findings. Chronic Bee Paralysis Virus (CBPV) was detected only in winter, with a prevalence of 12.5%. In contrast, Acute Bee Paralysis Virus (ABPV) and Deformed Wing Virus (DWV) were present in both seasons. ABPV prevalence was 2.5% in winter and 32.5% in spring, while DWV was found at 7.5% and 22.5% respectively. These findings are consistent with the global prevalence of these viruses. Many studies[30,31] show that the Varroa mite, the main parasite of honey bees, causes increased colony mortality by transmitting the dangerous DWV-B virus. In Iran, ABPV and CBPV were first reported by Moharrami and Modirrousta[32]. Statistical analysis revealed a significant correlation between viral factors and colony collapse disorder (CCD) only in spring ($p = 0.035$). This supports a complex relationship, as other studies have shown mixed results. This highlights that multiple factors, including beekeeping practices like migration and the specific genetic makeup of the bees, can influence CCD susceptibility.

A key aspect of this interaction is the synergistic effect of co-infection. Studies by Toplak *et al.*[33] demonstrated that co-infection with *Nosema ceranae* significantly increases CBPV replication and leads to premature bee death. This suggests that while a single pathogen might not cause CCD, their combined effect could be a primary driver. Hence, statistical analysis, however, did not find a significant correlation between co-infection with *Nosema*, *Varroa* and viral agents ($p>0.05$).

CONCLUSION

This study confirms that honey bee population decline is a complex issue with multiple contributing factors. Findings suggest that *Nosema ceranae* is a significant seasonal threat, with peak prevalence coinciding with the highest bee losses in winter and spring, though it may not be the sole cause of colony collapse. Furthermore, the absence of other key parasites like *Acarapis* mites indicates a unique local epidemiological profile. These results highlight the need to consider a combination of parasitic, viral and environmental factors, tailored to specific geographical contexts, to effectively manage and mitigate honey bee population decline.

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