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Molecular Examination of Pathogens in the Red Dwarf Honeybee, *Apis Florea*, in Southwest Saudi Arabia

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Abstract

Apis florea is a wild honeybee native to Asia that is currently spreading throughout the countries of the Middle East and East Africa, including Saudi Arabia. The spread of these honeybees raises questions regarding their pathogens, pests, and the diseases they may harbor. This is the first study of the prevalence of *A. florea* pathogens in Saudi Arabia. Samples from three different locations were diagnosed to identify six honeybee viruses: acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), deformed wing virus type A (DWV-A), deformed wing virus type B (DWV-B), and sacbrood virus (SBV); two microsporidian gut parasites, *Nosema apis* and *Nosema ceranae*; one fungi, *Ascosphaera apis*; two bacterial pathogens, *Paenibacillus larvae* and *Melissococcus plutonius*; and the ectoparasites *Euvarroa sinhai* and *Varroa destructor*. In this study, ABPV, CBPV, BQCV, DWV-A, *N. apis*, *N. ceranae*, and *M. plutonius* were detected in *A. florea*, while DWV-B, SBV, *A. apis*, *P. larvae*, *E. sinhai*, and *V. destructor* were absent. The findings of this study represent the initial contribution to understanding the presence of pathogens in the *A. florea* honeybee in Saudi Arabia. However, further research is necessary to develop sustainable strategies for the beekeeping industry and awareness of pathogens that cause diseases in the country.

Keywords: Apis florea, honeybee viruses, Nosema spp, Melissococcus plutonius, Saudi Arabia

Introduction

Honeybees (*Apis* spp., Apidae) represent the insect species most crucial to human beings, as they contribute significantly to the global pollination services essential to crop cultivation (Rader et al., 2016). Additionally, they are the source of bee-derived products including honey, wax, and pollen. Among honeybees, *Apis florea*, known as the red dwarf honeybee, holds the distinction of being the most diminutive species within the *Apis* genus (Lord and Nagi 1987). Indigenous to Asia, *A. florea* is a wild bee species with a distribution that spans from the low-lying regions of India to various parts of Southeast Asia (Hepburn and Radloff 2011, Saraithong et al. 2015, Özkan Koca et al. 2018).

Presently, the geographical range of *A. florea* is witnessing an expansion across nations in the Middle East and East Africa. Recent detections of *A. florea* have been reported in countries such as Ethiopia, Djibouti,

and Egypt (Moritz et al. 2010, Bezabih et al. 2014, Al-Kahtani and Taha 2014, Steiner 2017, Shebl 2017). These bees, found in subtropical regions, create a singular honeycomb structure in bushes, caves, and exposed areas rather than in cavities. They commonly construct combs around the branches of trees and shrubs (Nanork et al. 2005).

The red dwarf honeybee *A. florea*, identified as an invasive alien species, has drawn significant attention due to its extraordinarily aggressive proliferation through global import and export trade. The proliferation of invasive alien species indicates the extensive influence and impact of such species on biodiversity worldwide (Tian et al., 2023). Over thirty years ago, the open nested honeybee *A. florea*, was observed beyond its endemic range in Asia (El-Niweiri et al., 2019), marking a significant shift in its geographical distribution. *A. florea* was initially documented in the central region of Saudi Arabia in 1985, and since then, it has disseminated throughout the country (Alghamdi and Nuru 2013). The exact mechanism of its introduction remains uncertain, although it is speculated that it may have been transported through trade activities with Oman (Hroobi, 2020). *A. florea* exhibits a high degree of adaptability to hot and arid climates and has firmly established itself as an invasive wild bee species within Saudi Arabia (Alghamdi and Nuru 2013, Bhattacharyya et al., 2019). The success of this establishment underscores the resilience and adaptability of this species.

Honeybees have been identified as hosts to a multitude of pathogens. The most detrimental pathogens prevalent in honeybees include parasites, viruses, bacteria, microsporidians, and fungi (Cavigli et al., 2015; Matthijs et al., 2020; Ngor et al., 2020). These pathogens induce diseases that inflict substantial harm on the apiculture industry and can lead to significant economic losses on a global scale (Higes et al., 2008; Vanengelsdorp et al., 2010; Gisder and Genersch 2015). In instances where honeybees coexist in a sympatric region and share food resources, the potential for cross-infection is heightened (Seeley & Smith, 2015; Gómez-Moracho et al., 2017; Ngor et al., 2020).

There are complex dynamics of disease transmission within bee populations. For instance, pathogens harbored by one bee can be transmitted to others through various contact behaviors, such as the transfer of mites or competition for nectar resources (Tian et al., 2023). *A. florea* has been observed to exhibit strong competition with *Apis mellifera* during foraging activities, and there are instances where it might even pilfer resources from *A. mellifera* colonies (Chahal et al., 1986; El-Niweiri et al., 2005).

The potential diseases and pests that imported honeybees may carry are of significant concern. *A. florea* honeybees are known to harbor the parasitic mite *Euvarroa sinhai* (Koeniger et al. 1983). If these mites were to infest *A. mellifera* colonies, the outcomes would be uncertain and could potentially be as catastrophic as those observed with the infestation of *Varroa destructor* (Goulson 2003). It is well-documented that diseases can significantly expedite replacement by invasive species, especially if a disease is innocuous to an invading species but lethal to an indigenous species (El-Niweiri et al., 2019). Overall, the dynamics of disease transmission are complex and its potential impact on biodiversity is significant.

To date, there is limited knowledge regarding the prevalence of honeybee pathogens in the red dwarf honeybee *A. florea*. This study therefore investigates the effects of biotic stressors on this Asian red dwarf wild honeybee. This research aims to determine the presence of six specific honeybee viruses: acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), deformed wing virus type A (DWV-A), deformed wing virus type B (DWV-B), and sacbrood virus (SBV).

Furthermore, it seeks to identify the incidence of two types of honeybee eukaryotic parasites, namely, the microsporidia *Nosema apis* and *Nosema ceranae*; a fungus, *Ascosphaera apis*; two bacterial pathogens, *Paenibacillus larvae* and *Melissococcus plutonius*; and the ectoparasites *E. sinhai* and *V. destructor*. The study uses *A. florea* samples collected from Southwest Saudi Arabia. The wide variety of stressors for which this study screened indicates a comprehensive pathogens screening effort.

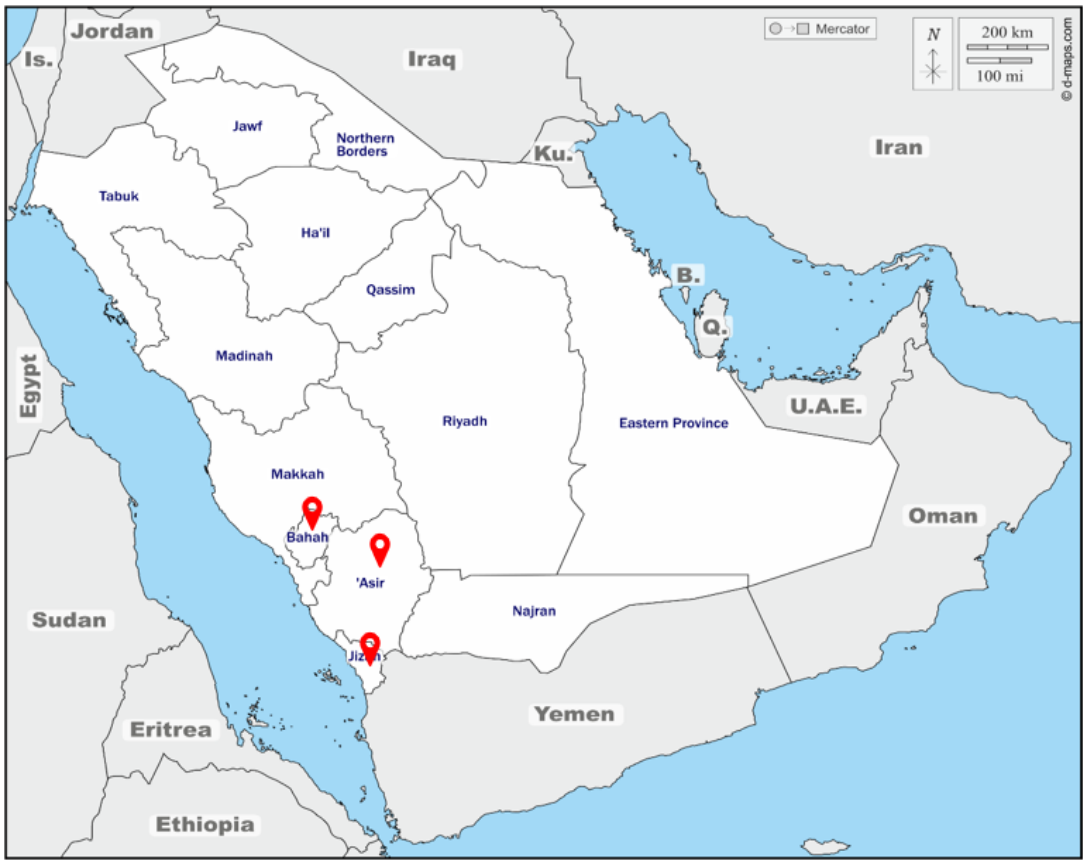

Figure 1. Locations of Sample Collection Sites of *A. florea* in Southwest Saudi Arabia.

Materials and Methods

Field Sampling

Three nests of *A. florea* were collected from three different regions (Al-Baha, Aser, and Jazan) in Saudi Arabia (Figure 1). The live bees were then immediately transported to a laboratory at Al-Baha University. Twenty adult workers in each nest were sampled and inspected for RNA viruses and other pathogens. All samples were stored at -80 °C prior to further examination in the laboratory. Additionally, approximately 400 adult worker bees obtained from each nest were deposited in a jar containing 70% ethanol to determine whether there was an *E. sinhai* or *V. destructor* infestation.

RNA Isolation and Reverse Transcription/cDNA Synthesis

Individual headless adult workers from each nest were homogenized using TRIzol reagent (Life Technologies) according to the manufacturer's instructions (Runckel et al. 2011). The RNA concentration and quality was evaluated using a NanoDrop Lite Plus spectrophotometer (Thermo Scientific, Loughborough. UK). The RNA samples were normalized to 500 ng/µL in RNase-free water and stored at -80 °C. The RNA was then reverse-transcribed into cDNA using TransScript reverse transcriptase (Transgene, China). To 5 µg of RNA, 1 µL of Oligo and nuclease-free water (variable) were added, and the mixture was incubated at 65 °C for 5 min to denature the RNA, after which the primers were allowed to anneal to the RNA. The sample was then placed on ice for 2 min, and 1 µL of dNTP mix (10 mM), 4 µL of RT buffer, 0.5 µL of ribonuclease inhibitor, and 1 µL of TransScript RT were added, and the mixture was incubated at 42 °C for 30 min, 25 °C for 10 min, and 85 °C for 5 s, followed by storage at -20 °C.

DNA Isolation for Microsporidia, Fungi, and Bacteria

The abdomen of 10 mature workers of the *A. florea* from each respective nest were collectively processed and homogenized. The extraction of the total genomic DNA was accomplished utilizing a Qiagen Tissue kit (Genomic DNA extraction kit, Germany), and the guidelines provided by the manufacturer were strictly adhered to. Prior to the commencement of the molecular screening for microsporidia, fungi, and bacteria, the DNA samples were preserved at a temperature of -20 °C.

Polymerase Chain Reaction (PCR)

Each specimen underwent a screening process for six RNA viruses: ABPV, CBPV, BQCV, DWV-A, VDV-1, and SBV, as well as five distinct honeybee pathogens: *N. apis*, *N. ceranae*, *A. apis*, *P. larvae*, and *M. plutonius*. The specific primers employed for the detection of the honeybee pathogens are enumerated in Table 1. The polymerase chain reaction (PCR) reactions were executed using My Cycler (Bio-Rad) in 20 µL volumes that were comprised of 1 µL of DNA or cDNA template, 10 µL of TransTaq High Fidelity (HiFi) PCR SuperMix (Transgene, China), and 1 µL each of forward and reverse primer, supplemented with 7 µL nuclease-free water. The thermocycler was set to run for 3 min at 94 °C, followed by 35 cycles of 30 s each at 94 °C, 30 s at a suitable melting temperature (Tm), 30 s at 72 °C, a final extension at 72 °C for 10 min. In each run, a PCR mixture devoid of DNA was utilized as a negative control. The amplicons obtained were subjected to electrophoresis on a 2% agarose gel to verify the size of the fragments with reference to a 100 bp ladder (Transgene, China).

Table 1. List of Primers Used in Molecular Detection of Pathogens in *A. florea* Honeybees.

N	Target	Primer	Sequence (5′ → 3′)	Size (bp)	Reference
1	ABPV	ABPV-F	TCTGATGATGCTGAAGAGAGAAA	500	Teixeira et al., 2008
		ABPV-R	AATCATCATTGCCGGCTCTA		
2	CBPV	CBPV-F	AGTTGTCATGGTTAACAGGATACGAG	455	Ribière et al., 2002
		CBPV-R	TCTAATCTTAGCACGAAAGCCGAG		
3	BQCV	BQCV-F	TGGTCAGCTCCCACTACCTTAAAC	700	Benjeddou et al., 2001
		BQCV-R	GCAACAAGA AGAAACGTAAACCAC		
4	DWV-A	DWV-A-F	GCGTGTTGCAACTCGCTTC	211	Bradford et al., 2017
		DWV-A-R	TGCCTGCACCGGATTCGATAAT		
5	DWV-B	DWV-B -F	GCAAGTTGGAGATAATTGTA	116	Moore et al., 2011
		DWV-B -R	CGATACTTACATTCTTCAAGAT		
6	SBV	SBV-F	TCGGATCCACCAAGTTGGAGG	349	Wang et al., 2015
		SBV-R	ACCTCATCACTCTGGGTCCTT		
7	*N. apis*	*N. apis*- F	CCATTGCCGGATAAGAGAGT	269	Chen et al., 2009
		N. apis-R	CCACCAAAAACTCCCAAGAG		
8	*N. ceranae*	*N. ceranae*-F	CGGATAAAAGAGTCCGTTACC	250	Chen et al., 2009
		N. ceranae-R	TGAGCAGGGTTCTAGGGAT		
9	*A. apis*	*A. apis*-F	TGTCTGTGCGGCTAGGTG	550	James & Skinner, 2005
		A. apis-R	CCACTAGAAGTAAATGATGGTTAGA		
10	*P. larvae*	*P. larvae*-F	AAGTCGAGCGGACCTTGTGTTTC	973	Garrido-Bailón et al., 2013
		P. larvae-R	TCTATCTCAAAACCGGTCAGAGG		
11	*M. plutnius*	*M. plutnius*-F	GAAGAGGAGTTAAAAGGCGC	831	Govan et al., 1998
		M. plutnius-R	TTATCTCTAAGGCGTTCAAAGG		

Detection of *E. sinhai* and *V. destructor*

The detection of *E. sinhai* and *V. destructor* were estimated using 70% ethanol, as described by Dietemann et al. (2013). The bees of each nest were investigated separately. Approximately 400 live adult *A. florea* honeybees were placed in a freezer for 20 minutes to cool. Shaking the bees in 70% ethanol for 3–5 min dislodged the mites from the bees. The washing process was repeated until no additional mites were detected on the adult bees.

Results

Virus Infections in *A. florea*

There is no previous record of the detection of RNA viruses and non-viral honeybee pathogens in the red dwarf honeybee *A. florea* in Saudi Arabia through the application of molecular identification techniques. Consequently, *A. florea* samples were collected from three different locations across Saudi Arabia and were analyzed using PCR. The RT–PCR data for six viruses in the non-cave honeybee *A. florea* honeybees are given in Table 2. Prevalence data for six honeybee viruses (ABPV, CBPV, BQCV, DWV-A, DWV-B, and SBV) were screened in three *A. florea* colonies from three locations. Honeybee viruses were detected in six pools of the three colonies. Of these viruses, only ABPV, CBPV, BQCV, and DWV-A were detected in *A. florea*. No DWV-B or SBV were found in *A. florea*. The band images, which were derived from the PCR process, are presented in Figure 2.

Table 2. Detection of Pathogens in *A. florea* Honeybees from Southwest Saudi Arabia**.**

Location	RNA viruses						Microsporidians		Fungi	Bacteria	
	ABPV	CBPV	BQCV	DWV-A	DWV-B	SBV	*N. apis*	*N. ceranae*	*A. apis*	*P. larvae*	*M. plutnius*
Al-Baha	+	+	+	+	-	-	+	+	-	-	+
	+	+	+	+	-	-	+	+	-	-	+
Asser	+	+	+	+	-	-	+	+	-	-	+
	+	+	+	+	-	-	+	+	-	-	+
Jazan	+	+	+	+	-	-	+	+	-	-	+
	+	+	+	+	-	-	+	+	-	-	+

Note. Abbreviations are as follows: DWV-A, Deformed Wing Virus A; VDV-1, varroa destructor virus-1; BQCV, Black Queen Cell Virus; SBV, Sacbrood Virus; ABPV, Acute Paralysis Virus; CBPV, Chronic Bee Paralysis Virus; *N. apis*, *Nosema apis*; *N. ceranae*, *Nosema ceranae; A. apis*, *Ascosphaera apis*; *P. larvae*, *Paenibacillus larvae*; *M. plutnius*, *Melissococcus plutonius*; +, positive sample; -, negative sample.

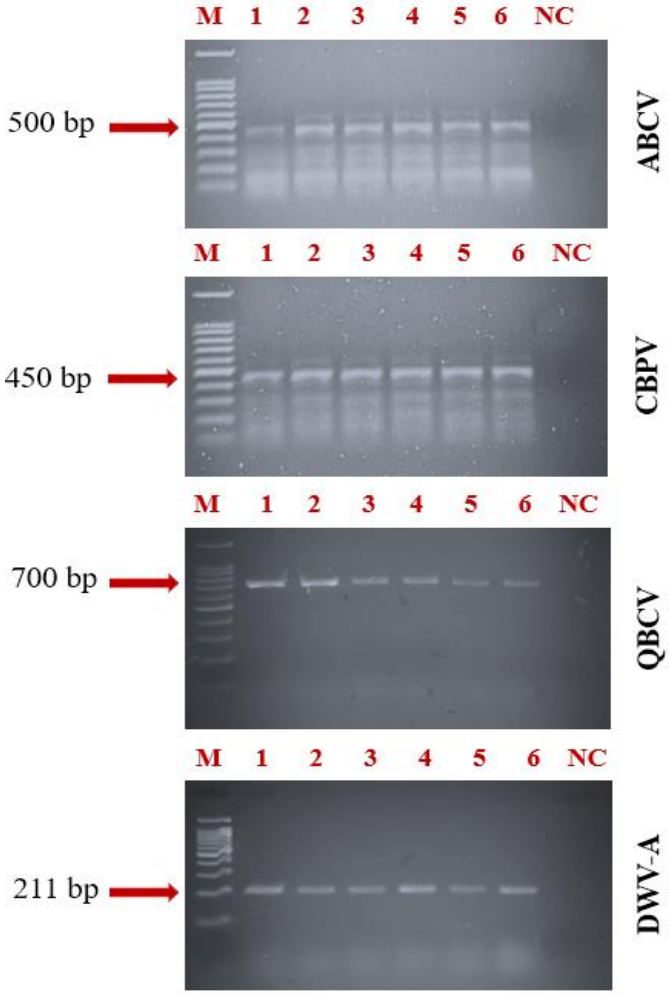

Figure 2. PCR Detection of RNA Viruses. Products amplified from total RNA were separated on a 2% agarose gel. A 100 bp DNA marker (M) is shown on the left. Lanes 1 to 6 represent honeybees infected by RNA viruses. Negative controls (NCs) were included in which water was added instead of the RNA template.

Detection of Non-viral Pathogens in *A. florea*

The *A. Florea* samples were DNA extracted and screened for microsporidia, fungi, and bacterial pathogens (*N. apis*, *N. ceranae*, *A. apis*, *P. larvae*, and *M. plutonius*). The results of the non-viral honeybee pathogen prevalence findings are summarized in Table 2. The microsporidia *N. apis* and *N. ceranae* were detected among all *A. florea* samples. The bacterium *M. plutonius*, the causative agent of European foulbrood, was found in the investigated *A. florea* samples across all locations. However, *A. apis* and *P. larvae* were not detected in any investigated nests of *A. florea* (Figure 2). *E. sinhai* or *V. destructor* were also not detected in any examined *A. florea* nests.

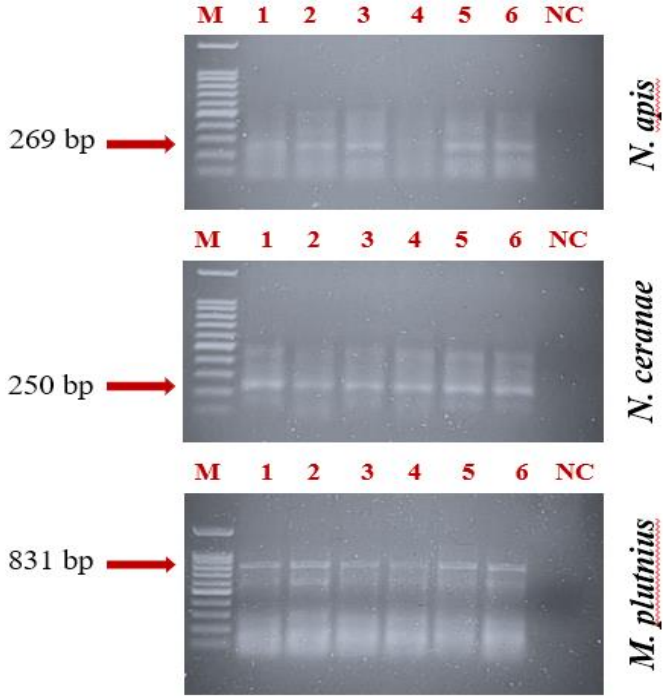

Figure 3. PCR Detection of Non-viral Honeybee Pathogens**.** Products amplified from total DNA were separated on a 2% agarose gel. A 100 bp DNA marker (M) is shown on the left. Lanes 1 to 6 represent *A. florea* bees infected by non-viral honeybee pathogens. Negative controls (NCs) were included in which water was added instead of the RNA template.

Discussion

The worldwide reduction in the population of bees that serve as pollinators has prompted a thorough investigation into host-microbiome communities (Potts et al., 2010). Honeybees, as significant agents of pollination, play an indispensable role in ensuring the yield of agricultural products and in preserving the balance of the ecosystem. In contrast to the western bee *A. mellifera*, *A. florea* is not cultivated for the commercial production of honey or wax but is found in the wild (Ganeshprasad et al., 2022). The principal contribution of *A. florea* honeybee species lies in their essential role in the pollination of a wide range of flowering plants and varied vegetation within tropical ecosystems (Shwetha et al., 2020). During their growth phase, honeybees typically encounter a multitude of pathogenic agents, including viruses, fungi, bacteria, and parasites. In addition, some of these pathogenic agents are known to be present in a wide range of hosts including managed and wild bees, mites, and ants (Sébastien et al. 2015; Campbell et al. 2016; Deutsch et al., 2023).

This study is among the first surveys in Saudi Arabia to examine honeybee pathogens in *A. florea* so that the knowledge of the diversity of pathogens found in *A. florea* can be expanded. The results revealed that ABPV, CBPV, BQCV, and DWV-A were present in adult *A. florea* samples from all sites, even though these honeybee samples presented no symptoms of disease. These results are consistent with previous studies indicating that ABPV, CBPV, BQCV, and DWV-A have been detected in wild bees (Gajger et al., 2021). For BQCV and DWV-A, this is not surprising, since they have been previously detected in *A. florea* (Zhang et al., 2012; Lin et al., 2021). However, this study reports the first detection of ABPV and CBPV in *A. florea*. The results of this study may indicate transmission of viruses from *A. mellifera* to *A. florea* because wild bees *A. florea* live near *A. mellifera* colonies and share flora. DWV-B and SBV were not detected in *A. florea* during this survey, but further testing should be conducted to provide more conclusive evidence of whether *A. florea* is free from these viruses.

Regarding the microsporidia, fungi, and bacterial pathogens evaluated in this study, *N. apis*, *N. ceranae*, and *M. plutonius* were detected in *A. florea*. However, *A. apis* and *P. larvae* were not detected in the *A. florea* samples. The detection of *N. ceranae* in *A. florea* was unsurprising due to its wide distribution and documentation across honeybees. The microsporidia of *N. apis* and *N. ceranae* are intracellular parasites that infect honeybees and other bees worldwide (Klee et al., 2007; Chaimanee et al., 2010; Han & Weiss, 2017). *N. ceranae* has been identified as the predominant species of Nosema affecting members of the *Apis* genus (Han & Weiss, 2017). For instance, *N. ceranae* in *A. florea* has been reported in Thailand in a similar study

to this one (Suwannapong et al., 2010). However, this study reports the first detection of *N. apis* in *A. florea*. This study also found the examined *A. florea* to be infected by the causative agent of European foulbrood, *M. plutonius*. This result is supported by a previous study in China that also reported that *M. plutonius* had infected the red dwarf honeybee *A. florea* (Lin et al., 2021). In Saudi Arabia, the beekeeping industry is severely threatened by European foulbrood (Ansari et al., 2017). Notably, *E. sinhai*, which is found as an ectoparasite in the original habitat of *A. florea*, and *V. destructor* were absent in any of the investigated colonies of *A. florea* in the three sites.

In conclusion, this research found that the non-cave honeybee *A. florea* located in various regions of Saudi Arabia were infected with multiple pathogens. The studied *A. florea* were positive for four viruses, ABPV, CBPV, BQCV, and DWV-A, but were negative for DWV-B and SBV. The present study also reports that the *A. florea* were infected by *N. apis*, *N. ceranae*, and *M. plutonius*, but *A. apis*, and *P. larvae* were not present in any of the analyzed samples. In addition, the ectoparasitic *E. sinhai* and *V. destructor* were absent within the investigated colonies. This study enhances the present understanding of *A. florea* colony health; however, further research is needed to further investigate the prevalence and infection level of these pathogens on a larger scale within Saudi Arabia.

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Data Availability Statement

Data will be made available on request.

Conflicts of Interest

The author declares no conflict of interest.

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