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# MICROSPORIDIAN *Nosema* spp. IN HONEY BEE COLONIES: ASSESSMENT OF INCIDENCE, MOLECULAR CHARACTERIZATION, AND ENVIRONMENTAL CORRELATIONS

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## ABSTRACT

Microsporidian parasites, *Nosema* spp. substantially affect the health of honey bee colonies. The current research provides extensive insights into the seasonal occurrence of Nosemosis in *Apis mellifera*, molecular identification of *Nosema* species, and its correlation with environmental factors. Of the two *Nosema* species (*Nosema apis* and *Nosema ceranae*), molecular identification using specific primers (321-APIS for *N. apis* and 218-MITOC for *N. ceranae*) confirmed the exclusive presence of *N. ceranae* in colonies of *A. mellifera* across three regions of Pakistan. *Nosema* incidence was categorized in three levels (I: 0-25%, II: 26-50%, and III: 51-75%) with Khyber Pakhtunkhwa (KPK) region showing the highest infection rates ranging from 16.6–75.0%, compared to Punjab (8.3–33.3%) and the Capital Territory (8.3–25.0%). Out of 300 colonies surveyed across all geographical regions, 101 colonies (33.66%) were found to be infected, with the highest prevalence observed in KPK (59.16%), followed by the Capital Territory (CT) at 18.33% and Punjab at 15.83%. Mean spore count per bee was highest in KPK (0.610 million), followed by the Punjab (0.396 million) and CT (0.349 million) regions. Winter, autumn, and spring seasons were conducive for Nosemosis prevalence in KPK (75%, 69.4%, and 49.9%, respectively), and winter, autumn and summer in Punjab (20.8%, 16.6%, and 16.6%, respectively). Summer and autumn in the CT region showed Nosemosis levels of 25.0% and 13.8%, respectively, with no cases reported during winter and spring seasons. Nosemosis showed positive significant correlations with relative humidity (0.78) in winter, and with distance to floral & water sources (0.90) in autumn. Beekeepers nationwide should monitor and manage *Nosema* infections to minimize colony impact.

**Keywords:** Honey bee; Apiaries; *Nosema ceranae; Nosema apis;* Nosemosis; Molecular diagnosis; Spore count; Seasons; Ecology.



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## INTRODUCTION

Honey bees produce many valuable products such as honey, royal jelly, propolis, pollen, beeswax and bee venom, known for their important medicinal properties (Viuda-Martos *et al.*, 2017; Alqarni *et al.*, 2021; Raweh *et al.*, 2022). Bees play significant role in pollinating numerous commercial crops (Suhail *et al.*, 2001a, b; Ahmad *et al.*, 2017; Hung *et al.*, 2018; Abuagla *et al.,* 2025). However, honey bees face continuous threat from various diseases and often exhibit disease symptoms (Iqbal, 2009; Chantawannakul *et al.*, 2016; Iqbal *et al.*, 2024), like early emergence of adult bees (Lecocq *et al.*, 2016), decreased larval feeding ability (Kang *et al.*, 1976), shortened life span (Goblirsch *et al.*, 2013), deformed wings (Iqbal and Mueller, 2007) and increased overwintering mortality, resulting in reduced colony productivity, fecundity, and viability (Botías *et al.*, 2013).

Nosemosis is a disease caused by a mycological microsporidian affecting adult honey bee workers, drones and queens (Fries, 1993). This intracellular pathogen belonging to the genus *Nosema*, comprises of three species: *Nosema apis*, *N. ceranae* and *N. neumanni* (Higes *et al.*, 2006; Chemurot *et al.*, 2017). *N. apis* is considered a serious etiological agent in European honey bee (*Apis mellifera* L.) (Higes *et al.*, 2006). Its spores are comparatively large with uniform oval bodies measuring 4-6 µm in length and 2-4 µm in width (Fries *et al.*, 1992),

and are tissue-specific, developing within the honey bee midgut epithelial cells (Liu, 1984; Huang and Solter, 2013). The spores of *N. ceranae* measure 4.4 × 2.2 µm (Utuk *et al.*, 2016). The impact of *N. neumanni* on host bees remained unreported (Botías *et al.*, 2012; Galajda *et al.*, 2021; Martín-Hernández *et al.,* 2018). Infection of *N. ceranae* and *N. apis* have been reported in various countries including Taiwan and China (Chen *et al.*, 2009), Turkey (Huang *et al.*, 2005), Jordan (Haddad, 2014), Saudi Arabia (Muz *et al.*, 2010; Haddad, 2014; Ansari *et al.*, 2017), United States (Chen *et al.*, 2008; Chen *et al.*, 2009), Europe and Vietnam (Higes *et al.*, 2006), and Thailand (Chaimanee *et al.*, 2010).

*Nosema* is primarily transmitted among bees orally through spore-containing food (Fries *et al.*, 1996), while vertical transmission occurs though mating between healthy queen and infected drones (Roberts *et al.*, 2015). Healthy bees often ingest spores from faeces of infected bees during Nosemosis, likely due to increase sweetness resulting from *Nosema*-induced gut metabolic changes that raise fermentative sugar byproducts in the faeces (Galajda *et al.*, 2021). Other transmission routes include poor beekeeping practices, contaminated wax foundation sheets, robbing of extensively infected hives or replacing the healthy queen with an infected one (Fernandez *et al.*, 2006). Within the first five days after ingesting infectious spores, the bee's gut wall collapses, intestinal epithelial cells lose plasma and a peritrophic membrane is impaired, causing gastric dysfunction, and poor digestion (Vidau *et al.*, 2014).

Nosemosis disease significantly affects the worker honey bees' health. Infected bees tend to become foragers at early ages (Martín-Hernández *et al.*, 2018). Older workers are more susceptible to infection than younger ones (Jack *et al.*, 2016), with a decrease in worker longevity by up to 40% (Higes *et al.*, 2006). The hypopharyngeal glands of infected bees secrete less royal jelly which impairs the feeding activity of the queen and young larvae (< 3 days old) (Marín-García *et al.*, 2022). The flight orientation of workers is negatively affected, reducing both duration and foraging distance (Wolf *et al.*, 2016). It also affects the longevity and reproductive health of drones (Traver and Fell, 2011). Sperms in older infected drones are reduced due to *N. apis* (Peng *et al.*, 2015), while body weight of drones is reduced due to *N. ceranae* (Retschnig *et al.*, 2014). The queens succumb to the disease during food exchange with infected workers, (Higes *et al.*, 2009), and compromising their reproductive health including the terminal oocytes (Liu, 1992).

Although the occurrence of *N. apis* is linked to winter bee mortality, the fundamental concern is the frequent existence of the disease without evident losses in the infected colonies. Severe symptoms are primarily documented in the temperate regions. The severity of infection may vary depending on bee subspecies, food supplies, agricultural practices in the vicinity, hive maintenance, and other abiotic and biotic factors (Fries *et al.*, 2013; Milbrath *et al.*, 2015). *N. ceranae* is more virulent than *N. apis*, resulting in significant population reduction of bees in the warmer regions of Europe (Higes *et al.*, 2008). Furthermore, the disease increases the susceptibility to chemical stresses due to a synergistic interaction with agricultural pesticides. Research suggest that *N. ceranae* is more devastating to honey bee colonies compared to *N. apis* (Gisder and Genersch, 2013).

To our knowledge, systematic research on the seasonal incidence and molecular identification of *Nosema* species infecting honey bees in Pakistan is not available. Beekeepers in Pakistan often report severe colony losses and symptoms resembling those of *Nosema* (Alam *et al.*, 2021). To bridge the gap in existing literature and given the severity of *Nosema* infection in Pakistan, this research aimed to investigate the seasonal incidence of *Nosema* in *A. mellifera* in different apiaries, identify the causative *Nosema* species through molecular methods, and examine the correlation between *Nosema* incidence and environmental conditions in prominent geographical regions of the country.

## MATERIALS AND METHODS

**Experimental site:** This study was conducted at three distinct geographic regions within Pakistan; Punjab, Khyber Pakhtunkhwa (KPK) and Islamabad (Capital Territory). The incidences of Nosemosis were systematically documented from 25 different apiaries over the two-year study period across 2020 to 2022 (Fig. 1 and Table 1). The identification of *Nosema* spores and subsequent species classification using molecular analysis was conducted at Bee Research Unit, Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi (32°17'48 N, 72°21'9 E).

**Bee Sampling:** Twelve bee colonies were randomly chosen from each apiary while walking diagonally, and a sample of 30 bees was collected from the entrance of each colony. The bees were carefully placed in labelled zip bags, promptly transferred and stored in the laboratory at -4 ºC for subsequent analysis. The data pertaining to the date of collection, location of apiary (Table 1), temperature, relative humidity, distance of the apiary from the water source and for food source were systematically recorded during the sampling process.

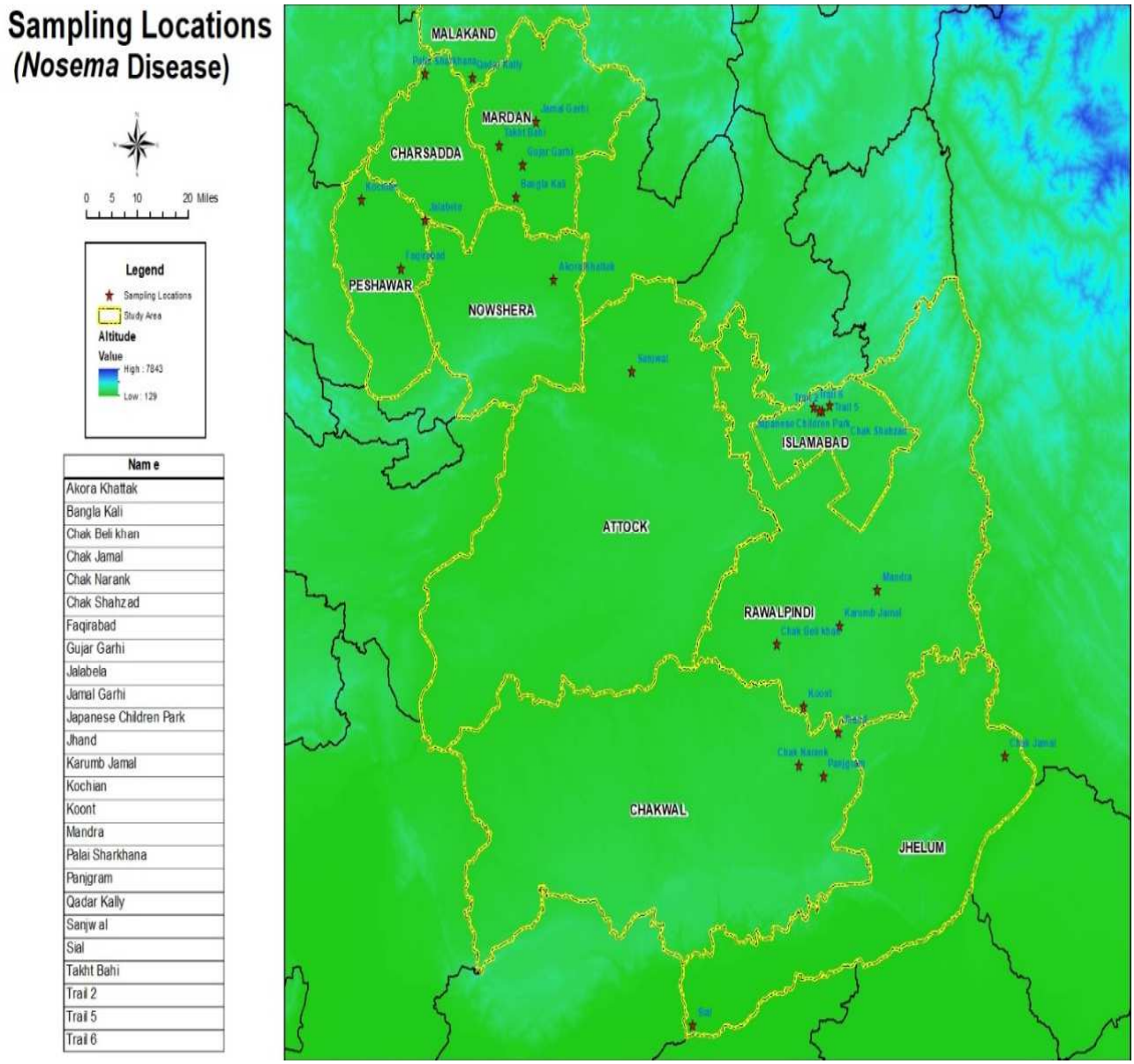
**Sample preparation and counting of *Nosema* spores:** Thirty bees from each collected sample were retrieved from the freezer and left at room temperature for 2 minutes. The abdomen of each bee was detached and homogenized in 15 ml distilled water (0.5 ml water per bee) using mortar and pestle. Subsequently, 10 µl of homogenized suspension from each sample was dispensed on Neubauer haemocytometer (Marienfeld,

German) and covered with a cover slip. Each sample was allowed to settle down on the haemocytometer for 90 sec before observing under a light microscope (Olympus Nea, Japan, 400x magnification). Three readings per sample were recorded, and the means were calculated to obtain the precise results regarding the incidence (absence/ presence) of *Nosema*. The disease incidence was classified into three categories: 0 to 25%, 26 to 50% and 51 to 75%, respectively. The number of spores from five squares of haemocytometer were counted according to the methodology of Reuter and Spivak (2019) using the following formula.

$$\text{No of spores observed from five squares} \times 25000 = \text{Million of spores /bee}$$

Here, 25,000 is the constant derived from chamber volume and grid dimensions.

**Molecular identification of *Nosema* spores:** Morphological characteristics of *N. cerana*e and *N. apis* were apparently similar, appearing oval with a dark outline and resembling a boiled egg. Consequently, to differentiate between two species of *Nosema* through microscopy proved challenging (Chen *et al.*, 2009; Milbrath *et al.*, 2015). Therefore, all positive bee samples exhibiting signs of *Nosema* incidence underwent Polymerase Chain Reaction (PCR) for the accurate identification of the particular *Nosema* species responsible for the infection.




| Name |
|---|
| Akora Khattak |
| Bangla Kali |
| Chak Beli khan |
| Chak Jamal |
| Chak Narank |
| Chak Shahzad |
| Faqirabad |
| Gujar Garhi |
| Jalabela |
| Jamal Garhi |
| Japanese Children Park |
| Jhand |
| Karumb Jamal |
| Kochian |
| Koont |
| Mandra |
| Palai Sharkhana |
| Panjgram |
| Qadar Kally |
| Sanjwal |
| Sial |
| Takht Bahi |
| Trail 2 |
| Trail 5 |
| Trail 6 |

**Figure 1: Map illustrating the collecting sites across three regions in Pakistan: Punjab, Capital Territory and Khyber Pakhtunkhwa. The red asterisk indicates the location of the sample collection site**

**Table 1. Characteristics of apiary sampling sites.**

| Location of sampling | | | | Geographical Coordinates | | | Sampling Season | |
|---|---|---|---|---|---|---|---|---|
| Regions | **Area/ city** | **Apiary Location** | | **Latitude** | **longitude** | **Altitude (m)** | **Season** | **Month -Year** |
| Punjab (Province) | Chakwal | PU-1 | Chak Narank | 33.003227 | 72.954915 | 504 | Winter | Jan-22 |
| | | PU-2 | Jhand | 33.061519 | 73.137911 | 457 | Autumn | Sep-21 |
| | | PU-3 | Chak Beli Khan | 33.236004 | 72.912097 | 475 | Summer | Jul-21 |
| | Rawalpindi | PU-4 | Karumb Jamal | 33.245203 | 73.211834 | 524 | Summer | May-21 |
| | | PU-5 | Koont | 33.116792 | 73.011123 | 524 | Winter | Nov-20 |
| | | PU-6 | Panjgram | 33.116792 | 73.011123 | 524 | Winter | Nov-20 |
| | | PU-7 | Mandra | 33.116792 | 73.011123 | 524 | Winter | Dec-21 |
| | Attock | PU-8 | Sanjwal | 33.761476 | 72.433951 | 402 | Spring | Apr-21 |
| | Jhelum | PU-9 | Sial Jhelum | 32.657394 | 72.2795 | 192 | Spring | Apr-21 |
| | | PU-10 | Chak Jamal | 33.020455 | 73.690409 | 252 | Winter | Nov-21 |
| Capital Territory | Islamabad | CT-1 | Chak Shahzad | 33.661694 | 73.143812 | 496 | Autumn | Aug-21 |
| | | CT-2 | Japanese Children Park | 33.743889 | 73.022778 | 1021 | Autumn | Oct-21 |
| | | CT-3 | Margalla Hills Trail 2 | 33.743889 | 73.022778 | 1021 | Summer | Oct-21 |
| | | CT-4 | Margalla Hills Trail 5 | 33.743889 | 73.022778 | 1021 | Autumn | Aug-21 |
| | | CT-5 | Margalla Hills Trail 6 | 33.743889 | 73.022778 | 1021 | Summer | Sep-21 |
| Khyber Pakhtunkhwa (Province) | Mardan | KP-1 | Faqirabad | 34.15437 | 72.100514 | 298 | Winter | Jan-21 |
| | | KP-2 | Jamal Garhi | 34.315801 | 71.067556 | 353 | Spring | Mar-21 |
| | | KP-3 | Gujar Garhi | 34.228024 | 72.020149 | 320 | Winter | Dec-21 |
| | | KP-4 | Bangla Kali | 34.228024 | 72.020149 | 323 | Winter | Oct-21 |
| | | KP-5 | Takht Bahi | 34.287572 | 71.934206 | 384 | Winter | Nov-21 |
| | Peshawar | KP-6 | Jalabela | 34.104756 | 71.934206 | 295 | Summer | May-21 |
| | | KP-7 | Kochian | 34.131226 | 71.46333 | 448 | Winter | Oct-21 |
| | Nowshera | KP-8 | Akora Khattak | 33.994958 | 72.135689 | 304 | Winter | Nov-21 |
| | Malakand | KP-9 | Palai Sharkhana | 34.530999 | 72.091012 | 426 | Winter | Oct-21 |
| | | KP-10 | Qadar Kally | 34.401474 | 71.84446 | 710 | Winter | Nov-21 |

**Purification and filtration of *Nosema* spores:** The samples underwent purification in 15 ml falcon tubes through centrifugation (80-2 Electronic Centrifuge, Pakistan) at 4000 rpm and room temperature for 5 min to eliminate the unwanted materials. Following centrifugation, the suspension was carefully discarded from each tube, and 1 ml of distilled water was added to each spore pellet. These samples were transferred to 1.5 ml Eppendorf tubes and centrifuged (D-LAB -D3024R; Micro-centrifuge, USA) at 8000 rpm for 10 minutes at 4°C. Once again, 1 ml of distilled water was added to the spore pellet in each Eppendorf tube, and vortexed (WizeMix vm-10) for 3-4 min. The mixture was then filtered through double cheese cloth and centrifuged at 8000 rpm for 10 min at 4°C. After discarding the suspension, the spore pellets were preserved at –20°C for subsequent analysis.

**DNA Extraction:** Genomic DNA was extracted from pooled *Nosema* positive bee samples using phenol and chloroform method as described by Hamiduzzaman *et al.* (2010). The spores within the samples were crushed in 300 µl DNA extraction buffer (comprising 0.03 M hexadecyl trimethyl ammonium bromide, 0.05 M tris hydroxymethyl aminomethane, 0.01 M ethylenediamine tetra-acetic acid, and 1.1 M NaCl), along with 500 µl of a solution (phenol: chloroform: isoamyl alcohol in 25:24:1 ratio). The resulting 500 µl sample was transferred to labelled 1.5 ml Eppendorf tube and centrifuged at 12,000 rpm for 10 min at 4°C. The upper layer of supernatant was carefully extracted and transferred to another labeled Eppendorf tube. In this tube, 500 µl chilled isopropanol and 50 µl chilled sodium acetate (pH: 4.8) were added, followed by centrifugation at 12,000 rpm for 10 min at 4°C. Afterwards, the suspension was discarded, and the DNA pellet was washed with an equal volume of 75% ethanol, and centrifuged at 10000 rpm for 10 min at 4°C. After discarding the suspension, the DNA pellet was mixed in $ddH_2O$ to make final volume of 50 µl and stored at –20°C for further analysis.

**Primers and PCR amplification of DNA:** The molecular characterization to identify *Nosema* spp. was conducted using PCR with specific primers designed to target *N. ceranae* and *N. apis* following the protocol outlined by Martín-Hernández *et al.* (2007) (Table 2). DNA was extracted from the pooled sample of infected bees.

**Table 2. Detail of specific primers for the molecular identification of *Nosema ceranae* and *N. apis***

| *Nosema* spp. | Primers Forward (F) Reverse (R) | Sequences (5' to 3') | Size (bp) |
|---|---|---|---|
| *N. ceranae* | 218-Mitoc-F | CGGCGACGATGTGATATGAAAATATTAA | 218-219 |
| | 218-Mitoc-R | CCCGGTCATTCTCAAACAAAAAACCG | |
| *N. apis* | 321-APIS-F | GGGGGC ATGTCTTTGACGTACTATGTA | 321 |
| | 321-APIS-R | GGGGGGCGTTTAAAATGTGAA ACAACTATG | |

The PCR was conducted for the samples using a thermal cycler (My-Gene-TM Series Peltier thermal cycler, Model MGG96G) to amplify partial 16S Small subunit rRNA genes of *N. apis* and *N. ceranae*. For each PCR reaction, Eppendorf tubes with a total final volume of 50 µl were prepared, containing 2.5 µl template DNA, 5 µl 10X PCR buffer. The PCR buffer comprised of 100 mM Tris–HCl, pH 8.3, 4 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100), 25.7 µl DNase and RNase free sterile distilled water, 5 µl 25mM $MgCl_2$, 6 µl dNTPs mix, 1 µl of forward and reverse primers, and 0.25 µl of Taq DNA polymerase (0.7 IU). The thermal cycling parameters for PCR consisted of an initial denaturation step (95$^o$C for 2 min), 35 cycles of annealing (95$^o$C for 60 s, 50$^o$C for 60 s, and 72$^o$C for 60 s), and final extension (72$^o$C for 5 min). Subsequently, the PCR products were subjected to e electrophoresis on a 1.5% agarose gel and the fragment sizes were determined using a 100 bp ladder (Cat No. M1200, Solarbio, Beijing, China). The amplified PCR bands were visualized and photographed using UV transilluminator (MS major Science USA) (Utuk *et al.*, 2016).

**Data analysis:** The incidence of Nosemosis was presented as percentage. Spore count data were subjected to analysis of mean and standard error using SPSS software. A two-tailed test was used to compare the mean number of spores among three regions. Additionally, a Pearson correlation was determined to assess the correlation between *Nosema* incidence and other factors such as date of collection, location of the apiary, temperature, relative humidity, and distance of apiary from the water and food source. The analysis was conducted using CoStat-Statistical Software Version 6.311. GraphPad Prism v.7 was used to construct graphical presentations of data.

## RESULTS

**Microscopic examination of bee midgut:** The midgut of the infected honey bee exhibited a swollen, white appearance lacking distinct rings, while the midgut of healthy bees appeared normal, ringed and brownish in color. Microscopic examination of the midgut contents from infected bees showed the presence of oval-shaped *Nosema* spores (varying in abundance) with dark outlines. Figure 2 presents the key diagnostic indicators of *Nosema* infection in *A. mellifera*. Visible symptoms of dysentery were observed at the hive entrance (Fig. 2A). A comparison revealed a distinct difference in midgut coloration, with infected bees exhibiting a whitish midgut in contrast to the brown midgut of healthy bees (Fig. 2B). Additionally, light microscopy at 400x magnification confirmed the presence of *Nosema* spores in midgut of infected bees (Fig. 2C).

**Incidence of *Nosema* in different localities:** The study comprised of 300 bee colonies across 25 different apiaries (10 apiaries located across each of the Punjab and KPK province, and 5 across the Capital Territory). Within all tested apiaries, the incidence of *Nosema* exhibited variation at different locations. Bee colonies were grouped according to the incidence of *Nosema* infection in each surveyed region based into three categories: Category I (0-25%), Category II (26-50%) and Category III (51-75%).

In Punjab, the level of *Nosema* incidence ranged from 8.3 to 16.6% (Category I) in the apiaries of eight locations, while incidences of 33.3% (Category II) were observed at remaining two locations (Table 3). The lowest incidence (8.3%) was recorded from five locations (PU-1, PU-3, PU-4, PU-8 and PU-9) followed by 16.6% from three locations (PU-2, PU-5 and PU-7). The highest incidence of *Nosema* (33.3%) was recorded in two locations (PU-6 and PU-10) (Table 3).

In Capital Territory, the incidence of *Nosema* ranged from 8.3% to 25.0% (Category I) across all five locations (Table 3). The highest infection (25.0%) was recorded at two locations (CT-4 and CT-5) followed by 16.6% at two locations (CT-2 and CT-3) and the lowest incidence (8.3%) at CT-1 (Table 3). *Nosema* infection exceeding 25% was not found at any location within the Capital Territory.

In KPK province, among ten surveyed locations, one location KP-1 showed lowest *Nosema* incidence of 16.6% (Category I), other location KP-4 (Bangla Kali-Mardan) had an incidence of 41.6% (Category II). The remaining eight locations had incidence ranging from 58.3% to 75.0% (Category III). The highest (75.0%) was detected from four locations (KP-3, KP-6, KP-5 and KP-7) (Table 3).

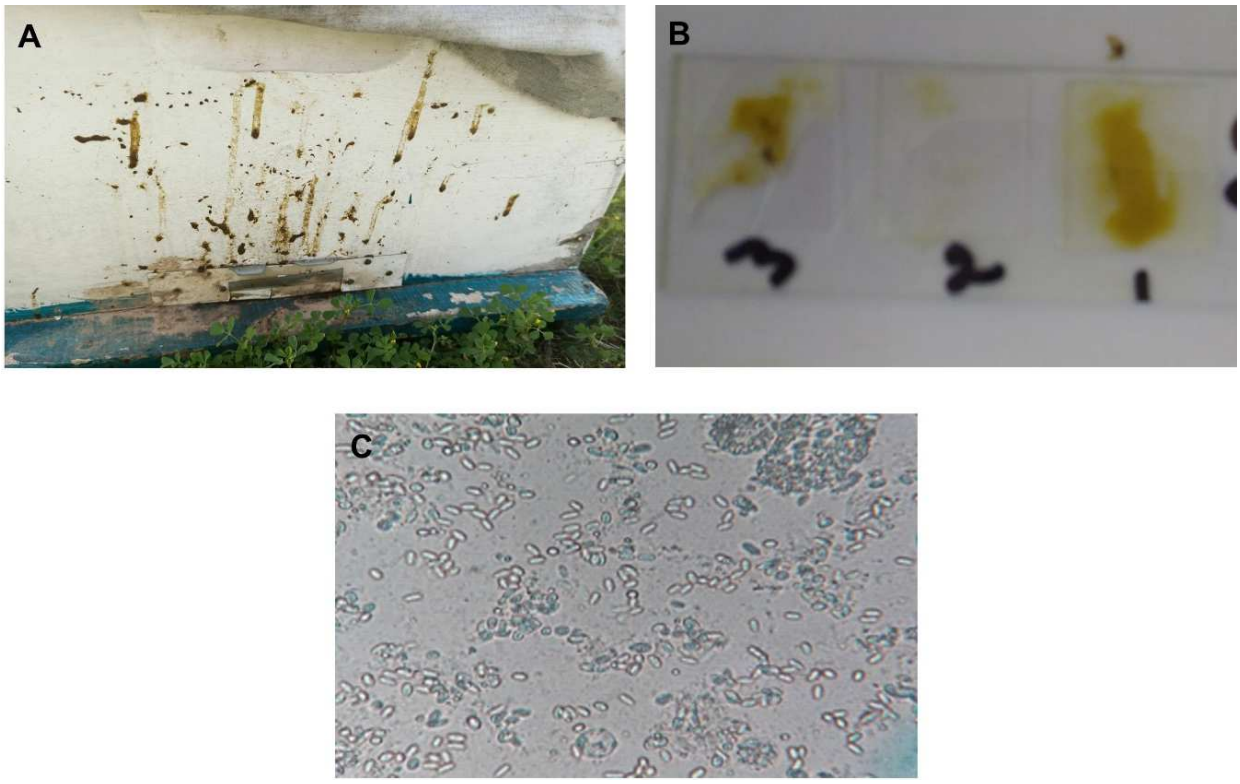



**Figure 2: Microscopic detection of *Nosema* from the midgut of honey bees. A) Symptoms of dysentery at the entrance of *Nosema* infected colonies B) Gut of healthy and infected honey bee: 1-Healthy bee; 2- Severe infection; 3- Mild Infection C) *Nosema* spores from the infected bees under light microscopy (400x magnification)**

**Table 3. Incidence of Nosemosis at different locations**

| Regions | | | Apiary Locations | Infection (%) | Infection Level* |
|---|---|---|---|---|---|
| Punjab | Chakwal | PU-1 | Chak Narank | 8.3 | Category-I |
| | | PU-2 | Jhand | 16.6 | " |
| | Rawalpindi | PU-3 | Chak Beli Khan | 8.3 | " |
| | | PU-4 | Karumb Jamal | 8.3 | " |
| | | PU-5 | Koont | 16.6 | " |
| | | PU-6 | Panjgram | 33.3 | Category-II |
| | | PU-7 | Mandra | 16.6 | Category-I |
| | Attock | PU-8 | Sanjwal | 8.3 | " |
| | Jhelum | PU-9 | Sial Jhelum | 8.3 | " |
| | | PU-10 | Chak Jamal | 33.3 | Category-II |
| Capital | Islamabad | CT-1 | Chak Shahzad | 8.3 | Category-I |
| Territory | | CT-2 | Japanese Children Park | 16.6 | Category-I |
| | | CT-3 | Margalla Hills Trail 2 | 16.6 | Category-I |
| | | CT-4 | Margalla Hills Trail 5 | 25.0 | Category-I |
| | | CT-5 | Margalla Hills Trail 6 | 25.0 | Category-I |
| Khyber | Mardan | KP-1 | Faqirabad | 16.6 | Category-I |
| Pakhtunkhwa | | KP-2 | Jamal Garhi | 58.3 | Category-III |

| | | | | |
|---|---|---|---|---|
| | KP-3 | Gujar Garhi | 75.0 | “ |
| | KP-4 | Bangla kali | 41.6 | Category-II |
| | KP-5 | Takht Bahi | 75.0 | Category-III |
| Peshawar | KP-6 | Jalabela | 75.0 | “ |
| | KP-7 | Kochian | 75.0 | “ |
| Nowshera | KP-8 | Akora Khattak | 58.3 | “ |
| Malakand | KP-9 | Palai Sharkhana | 66.6 | “ |
| | KP-10 | Qadar Kally | 66.6 | “ |

*Category I = 0 - 25%, Category II = 26-50%, Category III = 51 - 75% *Nosema* infection level.
“Refers to the above category

The collective average showed that 33.66% of bee colonies (101 out of 300 colonies) were found to be infected with *Nosema* spores. Specifically, in Punjab province, 15.83% (19 out of 120 colonies), Capital territory 18.33% (11 out of 60 colonies) and KPK 59.16% colonies (71 out of 120 colonies) (Table 4).

**Table 4. Cumulative incidence of Nosemosis from twenty-five different apiaries within the surveyed regions**

| Sampling region | Number of infected colonies | Total number of colonies | Infected colonies (%) |
|---|---|---|---|
| Punjab | 19 | 120 | 15.83 |
| Capital Territory | 11 | 60 | 18.33 |
| Khyber Pakhtunkhwa | 71 | 120 | 59.16 |
| Total | **101** | **300** | **33.66** |

**Counting of *Nosema* spores per bee:** In Punjab, the high number of *Nosema* spores per bee (in millions) was observed in the areas of Koont, (0.550±0.020), Mandra (0.470±0.000), Chak Jamal (0.435±0.065) and Panjgram (0.500±0.000). Conversely, the lowest spores count was recorded from the area of Karumb Jamal (0.200±0.000) (Fig. 3A).

In Capital Territory, the highest number of spores per bee was observed from Chak Shahzad (0.498±0.020), while the lowest spores were recorded from Margalla Hills Trail 2 (0.200±0.0001), and Japanese Children Park (0.200±0.0001) (Fig. 3B). In KPK, the highest number of spores per bee of *Nosema* was recorded from Jalabela (1.023±0.1248) followed by Bangla Kali (0.959±0.1147) and Gujar Garhi (0.846±0.1179), and minimum spores were observed in Qadar Kally (0.400±0.1000) (Fig. 3C). Collectively, the highest mean spore count (0.610 million) was found in KPK, followed by Punjab (0.396 million) and the Capital Territory (0.349 million) (Fig. 4).

**Seasonal incidence of Nosemosis:** The seasonal incidence of *Nosema* varied across all three regions (Punjab, Capital Territory and KPK**).** In Punjab, the incidence of *Nosema* was comparatively high in winter (20.8%), followed by incidences in both summer and autumn (16.6%), and the lowest incidence observed in the spring season (8.3%). In the Capital Territory, the incidence of *Nosema* was comparatively higher in summer (25.0%) than in the autumn season (13.8%), with no infection detected during the winter and spring seasons. In KPK, the incidence of *Nosema* was comparatively higher in winter (75.0%), followed by the incidences in autumn (69.4%) and spring (49.9%), while the lowest incidence was recorded in summer (16.6%). Collectively, the incidence of *Nosema* was much higher in all the seasons except in Punjab and Capital Territory for the spring season, and in Capital Territory for the winter season (Table 5).

**Pearson correlation of Nosemosis with other attributes:** The *Nosema* incidence showed a positive significant correlation (0.78) with relative humidity (RH) during winter and with distance from the flora & water (0.90) during autumn season. However, the correlation of *Nosema* incidence with all other attributes (temperature, RH, distance from the road, distance from the flora & water and distance from the population) was non-significant across tested seasons. Negative non-significant correlations were found for temperature during spring and summer (-0.46 and -0.43, respectively), RH during autumn (-0.92), distance from road during autumn (-0.33), and distance from population during spring and autumn (-0.50 and -0.52, respectively). Positive non-significant correlations were found for temperature during autumn and winter (0.33 and 0.37, respectively), RH during spring and summer (0.97 and 0.87, respectively), distance from road during spring, summer and winter (0.28, 0.53 and 0.26, respectively), distance from the flora and water during spring, summer and winter (0.92, 0.80 and 0.13, respectively), distance from the population during summer and winter (0.87 and 0.17, respectively) (Table 6).

**Molecular identification of *Nosema* spp.:** Multiplex-PCR using specific primers for *N. apis* and *N. ceranae* revealed the molecular identification of *Nosema* species in the infecting honey bees (Fig. 5). Agarose

electrophoresis gel (1.5%) under a UV transilluminator revealed a band size of 218-219 bp, corresponding to the presence of *N. ceranae*. Notably, only *N. ceranae* was detected in all the tested apiaries, while *N. apis* was not found in PCR analysis for the targeted apiaries (Fig. 5). Thus, only the prevalence of *N. ceranae* is confirmed from different geographical location of Pakistan.

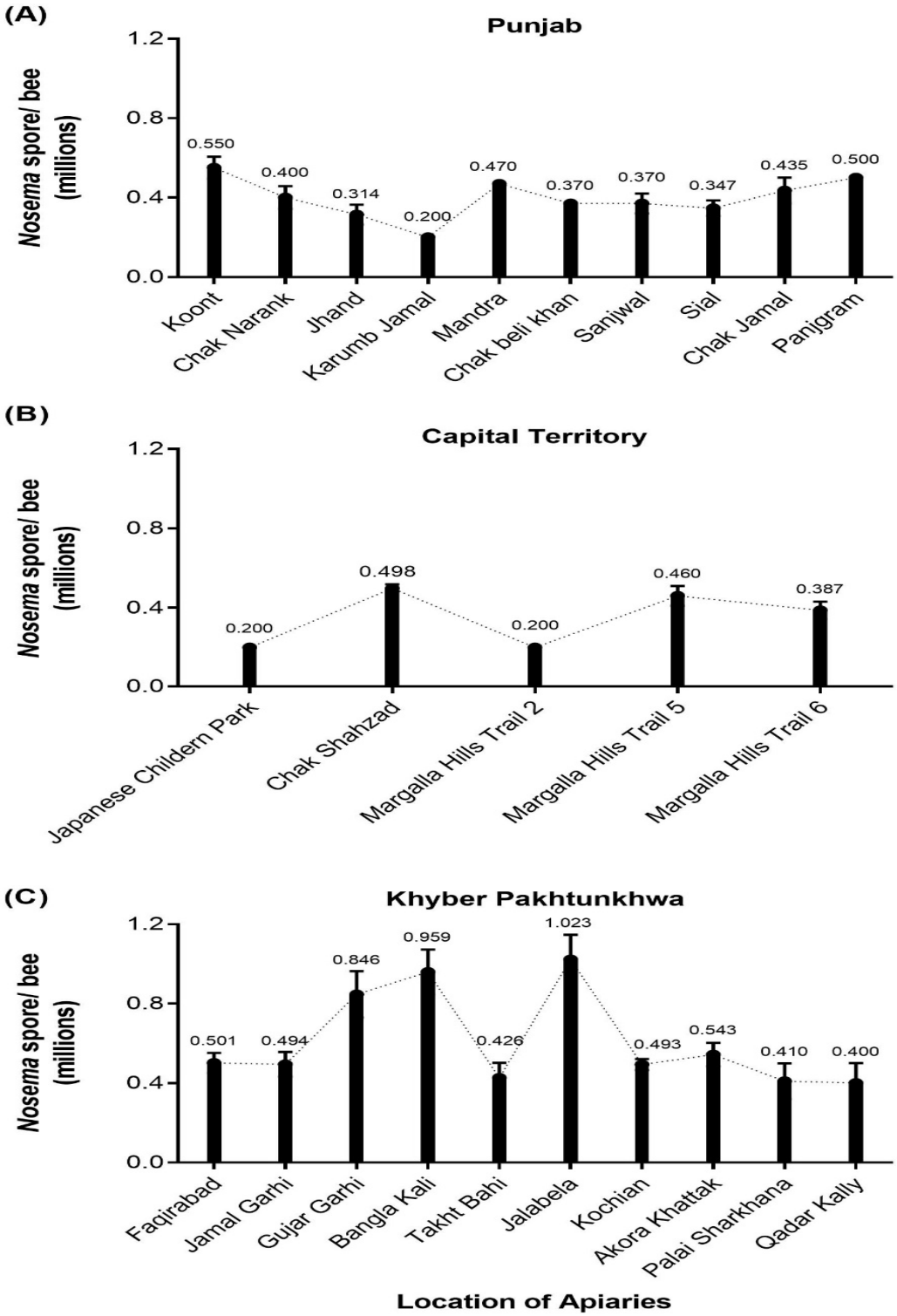



**Figure 3: Number of *Nosema* spores per bee (in millions) from apiaries in various geographical areas A) Punjab B) Capital Territory C) Khyber Pakhtunkhwa. The bars represent the standard error.**

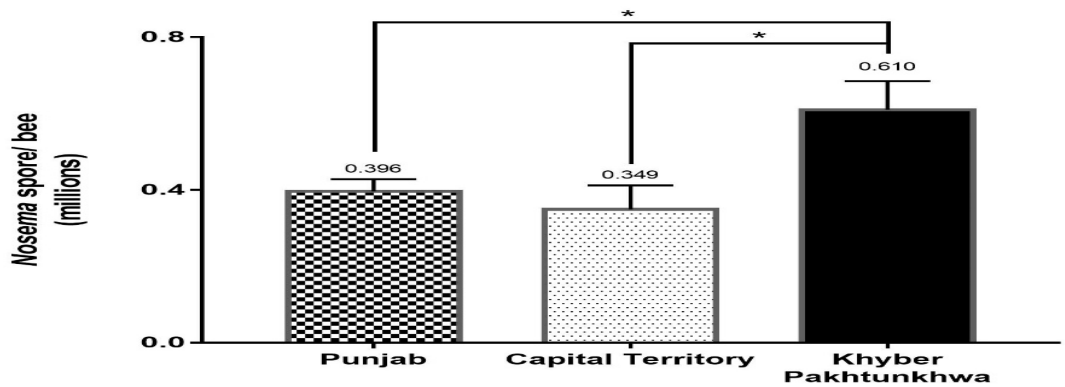



**Figure 4: Mean number of *Nosema* spores per bee (in millions) in three geographical regions of Pakistan. The bars represent the standard error and asterisks indicate the significant difference (Two tailed Test at $p < 0.05$).**

**Table 5. Seasonal incidence of Nosemosis in different regions of Pakistan**

| Regions | Seasons | Months | Mean Infection (%) |
|---|---|---|---|
| Punjab | Spring | March - April | 8.3 |
| | Summer | May - August | 16.6 |
| | Autumn | September - November | 16.6 |
| | Winter | December - February | 20.8 |
| Capital Territory | Spring | March - April | --- |
| | Summer | May - August | 25.0 |
| | Autumn | September - November | 13.8 |
| | Winter | December - February | --- |
| Khyber Pakhtunkhwa | Spring | March - April | 49.9 |
| | Summer | May - September | 16.6 |
| | Autumn | October - November | 69.4 |
| | Winter | December - February | 75.0 |

**Table 6. Correlation (r) of Nosemosis with various attributes in different regions of Pakistan**

| Seasons | Temperature (ºC) | Relative Humidity (%) | Distance from the road (Km) | Distance from the flora and water (Km) | Distance from the population (Km) |
|---|---|---|---|---|---|
| Spring | −0.46 | 0.97 | 0.28 | 0.92 | −0.50 |
| Summer | −0.43 | 0.87 | 0.53 | 0.80 | 0.87 |
| Autumn | 0.33 | −0.92 | −0.33 | 0.90 ** | −0.52 |
| Winter | 0.37 | 0.78 ** | 0.26 | 0.13 | 0.17 |

** = Highly significant

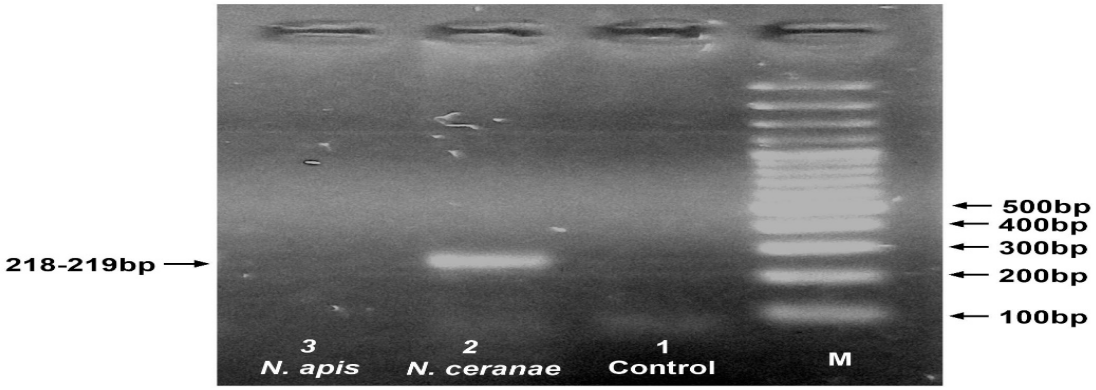



**Figure 5: PCR products amplified for the identification of *Nosema* species. M: marker, 1: negative control-distilled water (dd$H_20$), 2: PCR products for *Nosema ceranae* 218-219bp, 3: PCR products for *Nosema apis***

# DISCUSSION

Nosemosis represents a significant disease affecting honey bees (Martín-Hernández *et al.*, 2012), contributing to the decrease in number of bee colonies annually and impacting the honey production worldwide (Higes *et al.*, 2006; Paxton *et al.*, 2007). The disease's prevalence is closely linked to the environmental condition and geographic distribution of *A. mellifera*, raising concerns regarding potential variations in spore isolate virulence across different regions (Paxton, 2010; Martín-Hernández *et al.*, 2012; Mulholland *et al.*, 2012). Some perspectives suggest that *N. ceranae* is more competitive in warm climates, whereas *N. apis* are more adaptable to colder climates (Emsen *et al.*, 2016).

**Microscopic examination of bee midgut:** The colonies infected with *Nosema* exhibited symptoms of dysentery at the entrance of the hive, which is consistent with the findings from previous studies (Stevanovic *et al.*, 2013; Alattal and AlGhamdi, 2015). Upon dissection, the midgut of infected bees appeared white, swollen and without rings, which is in agreement with previous observations (Dar and Ahmad, 2013). The confirmation of *Nosema* spores presence was done through light microscopy (400x), which is consistent with methodologies outlined in earlier research ((Abdel-Baki *et al.*, 2016; Ansari *et al.*, 2017; Alam *et al.*, 2021).

**Incidence of Nosemosis:** We observed that 33.66% of the tested colonies were infected with *Nosema* spores, with high infection (incidence) noted during the winter season. Moreover, our investigation exclusively detected spores of *N. ceranae* in the tested bee colonies. However, studies in other regions have reported higher infection rates with *N. ceranae*, such as 67% in Saudi Arabia, 67.1% in Iran (Razmaraii *et al.*, 2013), and 60% in China (Yang *et al.*, 2013). In contrast, a study in India reported relatively less infection (18.1%) rate in colonies affected by *Nosema* spp. (Dar and Ahmad, 2013). A previous study in Pakistan utilized light microscopy to observe *Nosema* spp. spores in *A. mellifera*; however, the specific species of *Nosema* spores was not identified (Alam *et al.*, 2021). In contrast, our study specifically identified the species of *Nosema* spores.

The dual infection of *N. apis* and *N. ceranae* has been reported in bee colonies across different countries. In Turkey, 31% of bee colonies were dually infected with *N. apis* and *N. ceranae* (Utuk *et al.,* 2016), 52.2% of bee colonies in Siberia (Ostroverkhova *et al.*, 2019), and a total of 70.4% of colonies in Scotland (Bollan *et al.*, 2013). However, we did not find any evidence for the dual infection because only spores of *N. ceranae* were detected in the tested bee colonies.

**Counting of *Nosema* spores per bee:** *Nosema* spore counting per bee has typically been used to determine the level of infection within a colony (Furgala and Hyser, 1969). We found a high number of spores (>1,000,000 spores/ bee) in the honey bee samples collected from the KPK region (specifically Jalabela, Peshawar district). However, we could not find a relation between the number of *Nosema* spores and the percentage of infected colonies. In some colonies, the infection percentage was high with a minimum number of spores counting indicting that number does not correlate with the degree of infection. These findings align with conclusions of Higes *et al.* (2008), who discussed that the spore counting does not directly reflect the health status of colonies or the infection rate in colonies affected by *Nosema*.

**Seasonal incidence of Nosemosis:** The incidence of *Nosema* was detected throughout all four seasons of the year, aligning with the findings of Higes *et al.* (2010), who also observed *N. ceranae* infection across all seasons in honey bee colonies. Our study observed spring, winter and autumn as favorable seasons for the spread of *N. ceranae* in honey bee colonies in the surveyed regions of Pakistan. Likewise, high infection of *N. ceranae* in spring season was found in highly humid western and eastern Canada (Copley *et al.*, 2012). The elevated humidity was the primary factor driving the high infection during spring season. In our results, high infection percentage was found in different areas of KPK during spring, winter and autumn, likely due to the humid mountainous geographical position. In contrast, during the summer seasons, the infection rate was low, possibly due to high temperatures and low relative humidity. Reports from northwest Iran highlight a similar trend, with high *Nosema* infection during extremely humid spring seasons, creating favorable conditions for *Nosema* incidence (Topçu and Arslan, 2004; Martín-Hernández *et al.*, 2007; Lotfi *et al.,* 2009). Similarly, Kashmir, India, exhibited a higher infection rate during spring, and low infection rate during summer, speculated to be due to high humidity (Dar and Ahmad, 2013). Moreover, a high infection of *N. apis* was reported during spring and no infection during summer (Pickard and El-Shemy, 1989).

**Correlation of Nosemosis with environmental factors:** *Nosema* exhibits a strong relationship with environmental factors such as relative humidity, temperature, altitude, and flora, which directly influence the disease incidence (Martín-Hernández *et al.*, 2012). In our study, high incidence was found in different areas of KPK, likely attributed to the region's high-humidity mountainous geography. Conversely, during the summer season, the infection rate was low, possibly due to the combination of high temperatures and low relative humidity. These observations align with the findings of Özgör *et al.* (2015), who reported the direct impact of temperature and humidity on *Nosema* fluctuation. In addition, bee

colonies exhibited a high infection rate of *N. apis* in spring attributed to high humidity, contrasting with lower rates during the summer months characterized by high temperatures in Saudi Arabia (Ansari *et al.*, 2017), and a similar trend also observed in China (Wang *et al.*, 2019).

**Molecular identification of *Nosema* spp.:** The presence of *N. ceranae* was confirmed at 218-219 bp, while no band corresponding to *N. apis* was detected at 321bp. These results concluded the prevalence of only *N. ceranae* in the infected bees sampled from different geographical location of Pakistan. The band size of 218-219 bp for *N. ceranae* has been consistently reported in numerous studies worldwide. The mitoc-218-219 bp isolate exhibited an identical sequence of *N. ceranae* in Turkey (Utuk *et al.*, 2016). In Saudi Arabia, it was reported that the 218 bp isolate demonstrated a 100% correct sequence identity to *N. ceranae* (Ansari *et al.*, 2017). *N. ceranae* has been found to thrive in warmer climates, with the ability to survive at temperatures as high as 60°C (Fenoy *et al.*, 2009). It has been detected from *A. mellifera* colonies in different countries such as Taiwan (Huang *et al.*, 2005), Europe (Higes *et al.*, 2006), United States (Chen *et al.*, 2008; Chen *et al.*, 2009) and Japan (Yoshiyama and Kimura, 2011).

The present studies offer the initial insights into the seasonal incidence and molecular identification of *Nosema* in various geographical region of Pakistan. Through DNA-based molecular identification, these findings revealed for the first time that *N. ceranae* was most prevalent and virulent species across all the tested apiaries, compared to *N. apis*.

**Conclusion:** The prevalence of Nosemosis infection varied across seasons and geographical regions. It was high in winter and autumn, persisted through spring, and reached its lowest in summer, a trend likely reflecting the suppressive effects of high temperatures on pathogen development or host susceptibility. Molecular identification using DNA analysis revealed the exclusive presence of *N. ceranae* in all infected honey bee samples, with no detection of *N. apis* across all targeted geographical locations. It is suggested that beekeepers across the country adopt targeted and sustained management strategies against *Nosema* infection into their routine colony management to reduce associated colony losses.

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**Ethical statement:** Research and Ethics Committee of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi approved the field research as per Research Ethical and Conflict of Interest Policy 2021 of the University. Before sampling bees from hives, prior consent was obtained from the owner of the apiaries.

**Statement of conflict of interest:** The authors have declared no conflict of interest.

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