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PP-171 [Bee Health]

New *Nosema* multiplex qPCR reveals that 16SrRNA based PCR potentially overestimates *Nosema apis* prevalence and no correlation between viral load and *N. cer* in healthy beehives but a possible link in clinically affected colonies, in Austria

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Research Aim/INTRODUCTION

The honeybee midgut-infecting microsporidium *Nosema apis* (*N. apis*) was firstly described by Zander in 1909 in western honeybees (*Apis mellifera*), followed by *Fries* who discovered *Nosema ceranae* (*N. ceranae*) in 1996 - firstly appearing in eastern honeybee (*Apis cerana*) and in 2005 finally in *A. mellifera* (Huang, 2007). *Nosema* dates back in the last decade, but routine diagnostic detection still relies on microscopy and conventional PCR . Microscopy is time consuming and not as sensitive/specific as PCR . Conventional PCR situated in 16S ribosomal RNA (16SrRNA) represents an inadequate target for quantitative PCR because of multiple genomic copies per *Nosema* spore. The aim of this study is to **establish a sensitive multiplex *Nosema ssp.* qPCR** for accurate quantitation of *Nosema* spore load targeting a **single-copy genomic region** (DNA-dependent RNA Polymerase Subunit B1, RPB1). After encountering difficulties with multiplexing an already existing *Nosema ssp.* monoplex qPCR assay (Babin, 2022), we redesigned *N. ceranae* primers and probe. Additionally, we compared conventional PCR (Fries, 2013) with qPCR results. Concerning honeybee viruses, in literature knowledge on **interaction** between ***Nosema ssp.* and viral diseases** is scarce. We therefore compared honeybee viral load with *N. ceranae* spore load using our new qPCR. With this quantitative *Nosema ssp.* diagnostic tool, research on possible synergy between *Nosema* spore load and honeybee viral load is possible.

MATERIALS & METHODS

Overview of tested sample sets:

Honeybee viruses vs. *Nosema ssp.*

DATASET NR. + NAME	N	DESCRIPTION	LOCATION, YEAR
1 Historical sample set	139	Samples taken by ÖBGD within "Gesund Bienen II" project	All 9 federal states of Austria, 2022
2 Honeybee pathogen monitoring (standardized conditions)	62	Monitoring sampling in July and October	Graz (Austria), 2024
3 *Nosema* spp.- microscopical screening	22	Samples already microscopically examined for *Nosema* spores	2020, Austria
4 Citizen Sciences, honeybee virus monitoring	316	22 beekeepers have samples in May, June and September	Vienna (Austria), 2024
5 Winter loss monitoring in Vienna	87	Beekeepers reporting immense overwintering losses (2022/2023), mother hives + queen bees	Across all 23 districts of Vienna (Austria), 2023
6 Clinically affected CBPV honeybee colonies	6	Clinically apparent CBPV infected honeybee colonies (Diagnostic Purview Institute for Virology and Infectiology, Wiesbaden)	2023/24, Vienna (Austria)

Table 1: Overview of tested honeybee sample sets, a brief description

qPCR-assay design and performance

Primers and probe used for detection of *N. apis* derive from *Babin et al.* (2022). For ***N. ceranae***, **new primer and probe sequences** were designed using *DNAStrider* within same genomic region (DNA-dependent RNA-polymerase largest subunit B1, RPB1) where *N. apis* primers and probe were situated by Babin (2022).

Fig. 1: Alignment of Nosema ceranae/apis qPCR primers and probes within DNA-dependent RNA polymerase largest subunit B1 (RPB1) using GeneiousPrime®. Sequence shown in coloured bases resembles Nosema apis/ceranae RPB1 consensus extraction. Nucleotides marked in grey visualize mismatches (wobbles) between both Nosema species. Below consensus sequence, N. cer sequence is shown in upper case (NCIB Acc. No.: KF002468.1.) and N. apis sequence in lower case (NCIB Acc. no.: JX213665.1.). qPCR forward primers are highlighted in yellow and reverse primers in turquoise. qPCR probes are visualized in red. N. apis primers and probe are situated in upper row (Babin, 2022) and N. cer primers/probe in lower row.

qPCR assay was validated **in multiplex reaction**, detecting ***N. apis*** (Babin, 2022), ***N. ceranae*** (this study) and ***Apis mellifera* ß-actin** (internal control). Standard solutions (10^7 down to 10^1 copies) were tested for qPCR assay validation before testing samples. Limit of detection (**LOD**) for both *N. apis* and *N. ceranae* standards resulted in **10 copies per reaction**. For all qPCR reactions Luna® Universal Probe qPCR Master Mix Kit was applied. With each qPCR run performed, positive/negative controls, no-template-controls (NTC) as well as standard solutions (10^5 – 10^1 copies) were included.

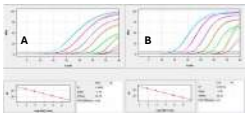

Fig. 2: N. apis (left) and N. ceranae (right) exponential standard dilution series curves and linear regression line (below each exponential standard curve diagram). Y-axis represents fluorescent dye (FAM for N. apis, Cy5 for N. ceranae) intensity and x-axis shows cycle of threshold (Ct). Validation of standard dilution series extending from 10^7 down to 10^1 copies was performed in triplicate reaction using Analytik Jena QTower3® qpcrsoft 4.1.

Conventional multiplex *Nosema ssp.* PCR (Fries, 2013) and RFLP (Gisder, 2010)

With datasets 1 - 3 conventional PCR in addition to qPCR was performed for validation. Additionally, a random sample set (n = 8) from dataset 1 was subjected to restriction enzyme analysis of *Nosema ssp.* as previously described by *Gisder et al.* (2010).

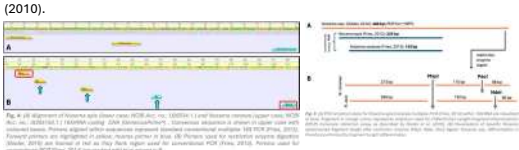

Fig. 4: (A) Alignment of Nosema apis (lower case; NCIB Acc. no.: JX213665.1.) and Nosema ceranae (upper case; NCIB Acc. no.: KF002468.1.) 16SRNA coding DNA (GeneiousPrime®). Consensus sequence is shown in upper case with coloured bases. Primers aligned within sequences represent standard conventional multiplex 16S PCR (Fries, 2013). Forward primers are highlighted in yellow, reverse primer in blue. (B) Primers used for restriction enzyme digestion (Gisder, 2010) are blamed in red as they flank region used for conventional PCR (Fries, 2013). Primers used for conventional PCR (Fries, 2013) are marked with blue arrows in B.

Fig. 5: (A) PCR amplicon for Nosema species-specific fragment (~1000 bp) of 8 samples (1-8) and positive control (+) and negative control (-). (B) Restriction enzyme digest of PCR amplicons with Mspl (CCGG) and Pael (CCTAGG). Fragment sizes of digested amplicons are indicated in base pairs (bp). Nosema apis (N.api) and Nosema ceranae (N.cer) are indicated.

RESULTS

Dataset 1, 2022 (n = 139)

qPCR
- 67 (**48%**) positive *for* ***N. cer***
- 1 (**0,7%**) positive for ***N. apis***

Conventional PCR (Fries, 2013)
- 50 (**36%**) positive for ***N. cer***
- 22 (**16%**) positive for ***N. apis* + *N. cer***
- 24 (**17%**) positive for ***N. apis***

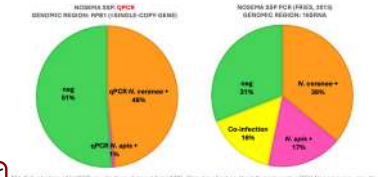

Fig. 6: Illustration of (q)PCR results from dataset 1 (n=139). Circular chart on the left represents qPCR Nosema ssp. results. On the right, results after conventional PCR testing (Fries, 2013) are shown. Both PCRs have primers situated in different genomic regions: conventional PCR primers are targeting 16SrRNA region (available in multiple copies) whilst qPCR assay is positioned in largest subunit B1 of the DNA- dependent RNA-polymerase II (single copy gene).

N = 8

RFLP (Restriction-Fragment-Length-Polymorphism; Gisder, 2010)
Sample nr. 047 (*N. apis* positive via qPCR) was verified after restriction enzyme digest as *N. apis* positive

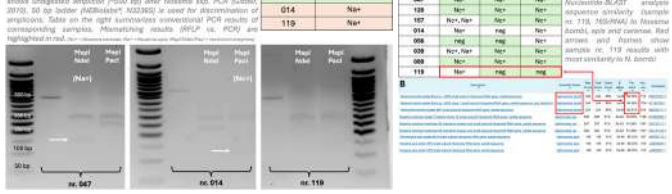

Fig. 6: Gel (3% agarose) image of 3 samples after performing Restriction-Fragment- Length-Polymorphism (RFLP) assay as described by Gisder et al. (2010). Sample nr. is highlighted below corresponding gel lanes. First lane shows undigested amplicon (~1000 bp) after Nosema ssp. PCR (Gisder, 2010). 50 bp ladder (NEBiolabs®, N3236S) is used for discrimination of amplicons. Table on the right summarizes conventional PCR results of corresponding samples. Mismatching results (RFLP vs. PCR) are highlighted in red.

Sample	Conventional PCR (Fries, 2013)
047	Nа+
014	Nа+
119	Nа+

Fig. 7: (A) Comparison of qPCR, conventional PCR and RFLP results (red). (B) NCIB® Nucleotide BLAST® analysis sequence similarity (sample nr. 119, 16SrRNA) to Nosema bombi, apis and ceranae. Red arrows and frames show sample nr. 119 results with most similarity to N. bombi

Dataset 2, 2024/25 (n = 62)

- 24 (39%) positive for *N. cer*, no *N. apis* detected
- prevalence: **July** = **31%**, **October** = **47%**
- 2 (**3,2%**) samples **undetected** via **qPCR**, detected via conventional PCR (Fries, 2013)

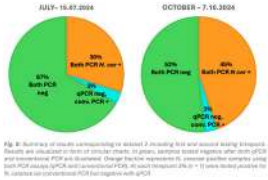

Fig. 8: Summary of results corresponding to dataset 2 including first and second sampling timepoint. Results are visualized in form of circular charts. In green, summed tested negatives after both qPCR and conventional PCR are illustrated. Orange (tactile) represents N. ceranae positive samples using both PCR assays (qPCR and conventional PCR). At each timepoint 2% (n = 1) were tested positive for N. ceranae via conventional PCR but negative with qPCR

Dataset 3, 2024 (n = 22)

8 samples (**36%**) were tested positive for ***N. cer*** via qPCR and conventional PCR (Fries, 2013). **Both PCR** results were **congruent**. Previously performed **microscopy** resulted in **3 samples not being detected** by microscopy and **2 samples** being identified as **false positive** in comparison to molecular detection.

Dataset 4, 2024 (n = 316)

Viral load vs. *Nosema* spore-load

- 36 (11%) positive for *N. cer*
- ***N. cer*** prevalence: **May** = **19%**, **June** = **14%**, **September** = **6,7%**
- **No significant correlation** (Spearman) for *Nosema* spore load and honeybee viral load (**ABPV**, **CBPV**, **DWV-B**, **SBV**)

Dataset 5, 2025 (n = 87)

BQCV
- 41% *N. cer* +
- 2/21 (9,5%) queens *N. cer* +
- 6 (6,9%) samples: BQCV viral load above 10^8 copies/bee
  → **5**/6 *N. cer* **co-infection**

Dataset 6, 2024 (n = 6)

CBPV
- **clinically apparent CBPV** infected honeybee colonies
- **4**/6 (66%) ***N. cer*** +
- *N. cer* spore load up to 10^4 copies/reaction

Refrences

Babin, A., Schurr, F., Rivière, M. P., Chauzat, M. P., & Dubois, E. (2022). Specific detection and quantification of three microsporidia infecting bees, Nosema apis, Nosema ceranae, and Nosema bombi, using probe-based real-time PCR. *European Journal of Protistology*, *86*. https://doi.org/10.1016/j.ejop.2022.125935

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PP-171 [Bee Health]

A highly sensitive multiplex qPCR-assay for discrimination of Nosema ssp in honeybees reveals that Nosema is no predisposing factor for the prevalence of ABPV, CBPV, SBV and DWV in Vienna, Austria

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Our indispensable pollinator – the honeybee (Apis mellifera) – is being threatened by various pathogens which often result in decreased performance capacity of worker bees and in worst case in colony losses. Common cause of winter-colony-losses is the infestation with varroa mites in combination with viral diseases. Nosema ceranae (N. ceranae) – an obligate intracellular microsporidium which proliferates in midgut-epithelial cells of the bee – is often discussed in the context of colony losses. In literature there is no consensus whether N. ceranae is a causative agent concerning losses of bee colonies or not. To enable a reliable quantitative diagnosis of N. ceranae, we designed a modified multiplex qPCR assay in this undergraduate student project – detecting both N. ceranae and N. apis simultaneously by targeting the DNA dependent RNA-polymerase II subunit B1 (RPB1). 20 apiaries in Vienna throughout three different timepoints (May, June, September) were monitored. While N. apis was absent in the tested sample-set, prevalences of N. ceranae were 20% in May, 14,5 % in June and 5% in September. Statistical analysis of our N. ceranae spore-load data in relation to viral load of bee viruses (ABPV, DWV, SBV, CBPV) revealed that none of the viral pathogens correlated significantly with N. ceranae infection. In conclusion, N. ceranae manifested regardless of co-analyzed bee viruses and showed higher prevalence in spring/summer (May, June). N. ceranae has efficiently replaced N. apis in apiaries in Vienna. Finally, the aim of this study is the establishment of a sensitive qPCR-assay for detection of Nosema ssp with the ulterior motif to help beekeepers in monitoring their Nosema-status and to report to the beekeeping community that certain bee viruses resulted in no predisposition for Nosema and vice versa