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# Prevalence of honeybee viruses in the Czech Republic and coinfections with other honeybee disease

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**Abstract:** Six bee viruses, which occur in *Apis mellifera*, were monitored in the Czech Republic between 2006 and 2009. Samples of larvae and pupae collected from hives where American foulbrood was detected were screened for bee viruses and in the 125 samples of larvae, there was no confirmed case of a larva infected with both American foulbrood and a bee virus. Of 145 samples infected with the protozoan *Nosema apis*, there were 23 cases of coinfections with the BQCV virus, 18 with the DWV virus and 11 with the ABPV virus. All coinfections with three or four viruses were also statistically significant apart from the one between ABPV with CBPV and DWV. The PCA ordination diagram indicates that BQCV occurs mainly with *Nosema apis* and DWV mainly with ABPV.

**Key words:** *Nosema apis; Nosema ceranae; Paenibacillus;* DWV; ABPV; BQCV; SBV; KBV; CBPV

## Introduction

Under certain circumstances, honeybee colonies are infected by pathogens, which reduce the size of the colonies or cause their collapse. Why they collapse is unknown. It is suggested that it might due to a combination of diseases or a combination of diseases and other harmful factors in the environment. Viruses, bacteria, microsporidia and the parasitic mite *Varroa destructor* Anderson et Trueman, 2000 are all important parasites of honeybees (Schmidhempel 1995).

Currently, more than 15 honeybee viruses belonging to the families Picornaviridae and Dicistroviridae and consisting of a 30 nm isometric particles containing a single positive RNA strand have been characterized (Bailey 1967, 1976). These viruses include SBV and DWV (genus *Iflavirus*), and KBV, BQCV and ABPV (genus *Cripavirus*). The chronic bee paralysis virus (CBPV) remains unclassified (Ribiere et al. 2007). Epidemiological data suggest that these viruses have spread worldwide. This was facilitated by worldwide trade in and movement of bee colonies as well as by the vector of the viruses, the ectoparasitic mite *V. destructor* (Sammataro et al. 2000; Bakonyi et al. 2002). This hematophagous mite is a vector of viral diseases and a potential activator of several bee viruses, attacking both mature and immature bees (Chen et al. 2006; Chen & Siede 2007). Techniques for the routine screening of pathogens, which are based on PCR, have been developed (Genersch 2005; Chantawannakul et al. 2006; Lanzi et al. 2006; Parrella et al. 2006; Blanchard et al. 2008; Kukielka et al. 2007; Zhang et al. 2007). The presence of viruses in bee colonies is studied with respect to clinical problems. Regarding infections we can also assume latent presence, which is asymptomatic.

*Nosema apis* belongs to a group of opportunistic intracellular spore-forming parasites, which cause an illness in mature bees (*Apis mellifera* L., 1758) called nosema disease, which has spread worldwide (Webster et al. 2008). Until recently, the nosemosis disease was associated only with the microsporidia of *N. apis*, (Bailey 1955; Woyciechowski & Krol 2001) and recorded only in the honeybee, *A. mellifera*. This disease causes both a weakening and reduction in the size of bee colonies. In 1996 a new type of microsporidian, *N. ceranae*, a parasite of *Apis cerana* F., 1793 and morphologically similar to *N. apis*, was identified. Place of infection and reproduction of both parasites are the ventricular cells of both *A. mellifera* and *A. cerana*. Cross-infection experiments using caged worker bees have shown that *N. ceranae* is more pathogenic than *N. apis* (Higes et al. 2007; Paxton et al. 2007) and *N. ceranae* develops better in *A. mellifera* than in *A. cerana* (Forsgren & Fries 2010). Recently, there has been an increase in the number of reports of infections by microspodia in many European countries, which have resulted in signif-

Table 1. Oligonucleotides.

| Agent | Forward primer (5' to 3') | Reverse primer (5' to 3') | Length (bp) | T[a] (°C) | References |
|---|---|---|---|---|---|
| DWV | TTTGCAAGATGCTGTATGTGG | GTCGTGCAGCTCGATAGGAT | 395 | 48 | Stoltz et al. (1995) |
| SBV | GGATGAAAGGAAATTACCAG | CCACTAGGTGATCCACACT | 426 | 48 | |
| CBPV | AGTTGTCATGGTTAACAGGAT | ACGAG TCTAATCTTAGCACGAAAGCCGAG | 455 | 48 | Ribiere et al. (2002) |
| ABPV | TGAGAACACCTGTAATGTGG | ACCAGAGGGTTGACTGTGTG | 452 | 48 | |
| BQCV | GGACGAAAGGAAGCCTAAAC | ACTAGGAAGAGACTTGCACC | 424 | 48 | |
| KBV | GATGAACGTCGACCTATTGA | TGTGGGTTGGCTATGAGTCA | 3936 | 48 | Shimanuki et al. (1994) |
| PL | GCTCTGTTGCCAAGGAAGAA | AGGCGGAATGCTTACTGTGT | 451 | 55 | Bakonyi et al. (2003) |
| NA | GGGGGCATGTCTTTGACGTACTATGTA | GGGGGGCGTTTAAAATGTGAAACAACTATG | 218 | 62 | Martin-Hernandez et al. (2007) |
| NC | CGGCGACGATGTGATATGAAAATATTAA | CCCGGTCATTCTCAAACAAAAAACCG | 321 | 62 | Martin-Hernandez et al. (2007) |

Explanations: [a] Annealing temperature; NA – *Nosema apis*, NC – *Nosema ceranae*; PL – *Paenibacillus* larvae; DWV – Deformed wing virus; ABPV – Acute bee paralysis virus; SBV – Sacbrood virus; CBPV – Chronic bee paralysis virus; BQCV – Black queen cell virus; KBV – Kashmir bee virus.

icant losses of bees and the collapse of entire colonies in the field and at particular apiaries (Martin-Hernandez et al. 2007; Higes et al. 2008). As it is very difficult to distinguish between the two nosema diseases morphologically, a fast and precise technique based on a PCR reaction and differences in the nucleotide sequence of a partial large subunit ribosomal RNA was developed (Martin-Hernandez et al. 2007; Bourgeois et al. 2010; Chen et al. 2009).

American foulbrood (AFB) is a bacterial disease, which attacks the larvae of bees (*A. mellifera*). The seriousness of this disease is due to the ease with which it is transmitted, longevity of the spores of the pathogenic agent, *Paenibacillus larvae,* and last but not least, a high infection rate in young larvae. *P. larvae* can be identified using several methods (de Graaf et al. 2006). The most efficient is to use PCR to detect it in honey, larvae, pupae or beehive-debris (Govan et al. 1999; Dobbelaere et al. 2001; Bakonyi et al. 2003; Alippi et al. 2004; Ryba et al. 2009). This method is mainly based on the amplification of the 16S rRNA of *P. larvae*, which is used as the target gene for PCR. The objective of this work was to diagnose pathogens and their combinations in samples from diseased bee colonies in the Czech Republic.

## Material and methods

*Samples*

A total of 513 samples of mature bees, mites and brood from bee colonies with suspected pathological problems were sent in by beekeepers or veterinary services in the Czech Republic. All samples were stored at –70 °C until processing in the period between 2006 and 2009. Altogether, there were 388 samples of mature dead bees, used to determine presence of viruses and nosema disease, and 125 of larvae, used to determine presence of viruses and American foulbrood.

*DNA and RNA extraction*

The abdomen of the dried bees, pupae or whole larvae (depending on the agent to be detected) were macerated for 5 h at 4 °C in a tube and then crushed by shaking them in test tubes containing glass beads (ø 3 mm) and 2 ml of distilled water. Then 700 µl of the ground mixture was transferred to a filtration column and centrifuged at 1,000 rpm.

Then DNA was isolated from 250 µl of the filtrate using QIA DNA Mini Kit (Qiagen) following the manufacturer's instructions. The DNA was eluted into 50 µl and stored at –70 °C.

Similarly a further sample of 250 µl of filtrate was used for isolating RNA. Viral RNA was isolated by using a QIAamp RNA Mini Kit (Qiagen) and following the manufacturer's instructions. The RNA was eluted into 25 µl and stored at –70 °C. The cDNA was prepared according to manufacturer's instructions using MMLV reverse transcriptase (Fermentas) primed with random hexamers. The cDNA samples were used as a template for RT-PCR with primers specific to the virus gene.

*PCR*

The sequence of the primer, orientation, annealing temperature, lengths of final products and references are shown in Table 1. Desalted primers were obtained from Invitrogen (UK). Two U of HotStart Polymerase (Fermentas) in the manufacturer's buffer with 2.5 mM $MgCl_2$, 200 µM dNTPs and 1 µM primers were used to amplify 1.5 µl of cDNA (or DNA) in a total volume of 50 µl. The cycling conditions were: 1 min at 95 °C, 35 cycles of denaturing at 94 °C for 30 s, annealing (Table 1) and extension at 72 °C for 1 min. The temperature of the PCR reaction was increased at the end up to 72 °C for 8 minutes and then cooled to 4 °C. PCR products were separated using agarose (1.0%) gel electrophoresis, stained with ethidium bromide and photographed. Approximate product size was determined using the 100-bp molecular size ladder.

*Statistical analyses*

A chi-square test was performed for all combinations of the viruses that attack larvae and bees (NA, NC, ABPV, DWV,

Table 2. Number of positive samples.

| Year | NA | NC | PL | ABPV | BQCV | SBV | KBV | CBPV | DWV |
|---|---|---|---|---|---|---|---|---|---|
| 2006 | 5 | 0 | 4 | 0 | 1 | 0 | 0 | 0 | 3 |
| 2007 | 24 | 0 | 21 | 2 | 2 | 0 | 0 | 0 | 33 |
| 2008 | 92 | 7 | 21 | 43 | 32 | 18 | 0 | 3 | 53 |
| 2009 | 12 | 12 | 11 | 22 | 9 | 2 | 0 | 10 | 32 |
| Total | 133 | 19 | 57 | 67 | 44 | 20 | 0 | 13 | 121 |

Explanations: NA – *Nosema apis*; NC – *Nosema ceranae*; PL – *Paenibacillus* larvae; DWV – Deformed wing virus; ABPV – Acute bee paralysis virus; SBV – Sacbrood virus; CBPV – Chronic bee paralysis virus; BQCV – Black queen cell virus; KBV – Kashmir bee virus.

Table 3. Frequencies of coinfections.

| | 2006 | 2007 | 2008 | 2009 | Total |
|---|---|---|---|---|---|
| 0[a] | 13 | 32 | 76 | 28 | 149 |
| 1 | 9 | 54 | 165 | 59 | 287 |
| 2 | 2 | 6 | 39 | 14 | 61 |
| 3 | 0 | 0 | 7 | 6 | 13 |
| 4 | 0 | 0 | 1 | 2 | 3 |
| Total | 24 | 92 | 288 | 109 | 513 |

Explanations: [a] number of pathogens recorded in a sample.

BQCV, CBPV and SBV) to look for deviations from the expected occurrence of the viruses ($2 \times 2$ table for coinfections with two viruses, three-dimensional table for coinfections with three viruses and four-dimensional table for coinfections with four viruses). Bonferroni correction was applied in all multiple chi-square tests.

A principal component analysis (PCA) of virus coinfections was performed using CANOCO software (ter Braak & Smilauer 2002). For this analysis data from all years (2006–2009) and agents that attack bees and also larvae (microsporidia *N. apis* and *N. ceranae*; viruses ABPV, DWV, BQCV, CBPV and SBV), were used.

## Results

The samples analyzed in this study were collected from bee colonies suffering from non-specific problems, they were weak due to a great loss of bees or they had collapsed because of an unknown apparent reason.

During spring and summer there were some cases of bees with hairless abdomens crawling around the hives and during autumn the sudden collapse of entire colonies was reported. Of the 513 samples from colonies that were thought to be infected with pathogens, 410 (80%) were shown to be positive by the PCR analysis. Of the 388 samples of bees 145 (38%) were positive for nosemosis disease and 218 (56%) for bee viruses. Of the 125 samples of larvae, 47 (38%) were positive for American foulbrood (Table 2).

Of the 388 samples tested for bee viruses most (121; 31%) were infected with DWV, which accounts for 46% of the 265 viral infections. ABPV was the second most frequent virus in the samples (17%). The percentage incidence of the next three viruses in rank order was BQCV (11%), SBP (5%) and CBPV (3%). None of the samples collected between 2006 and 2009 contained the KBV virus. No virus, nosemosis either *P. larvae* were detected in 29% (148) of all the samples that were suspected of containing pathogens. This might be due to either intoxication or other factors causing the death of the bees.

Samples coinfected with 2 of the 9 pathogens made up 11.7%, those coinfected with 3 pathogens 2.5% and with 4, 0.5% of all the samples (Table 3). Another aspect of this research was the frequency of coinfections with AFB and viruses. American foulbrood attacks the larvae of bees and nosemosis only mature bees. For this reason, neither larvae nor brood were tested for nosemosis disease.

Chi-square tests indicate that the significantly most common co-infections were the ones between *N. apis* and BQCV or *N. apis* and DWV. Co-infection between three or four viruses were also significant apart the one between ABPV with CBPV and DWV (Tables 4, 5).

The PCA ordination diagram (Fig. 1) indicates that the virus BQCV occurred mainly as a coinfection with *N. apis* and DWV with ABPV, whereas CBPV, SBV and the microsporidian, *N. ceranae,* occur mostly separately.

## Discussion

The PCR method has become the standard, reliable, rapid, specific and accurate method for detecting many pathogens. There is no doubt that new matrix sample analysis, targeted primers and multiplex methods will increase the efficiency and reduce the expense per sample analyzed. The routine screening for pathogens currently plays a valuable role in the detection of diseases, detection of diseases in pre-clinical stages and prevention of losses.

Globalization of trade provides the means by which both parasites and pathogens are transported across

Table 4. Frequencies of simultaneous viral and nosemosis infections in the 513 samples of honeybees.

| No. of viruses | Type of coinfection | No. of samples | Percentage |
|---|---|---|---|
| 2 | ABPV, DWV | 12 | 2.87 |
| | ABPV, CBPV | 1 | 0.23 |
| | ABPV, BQCV | 3 | 0.72 |
| | NA, BQCV | 18 | 1.68 |
| | NA, DWV | 10 | 2.30 |
| | NA, ABPV | 5 | 1.15 |
| | NA, NC | 3 | 0.72 |
| | ABPV, SBV | 2 | 0.46 |
| | BQCV, DWV | 2 | 0.46 |
| | SBV, DWV | 1 | 0.23 |
| | BQCV, SBV | 1 | 0.23 |
| | NC, DWV | 1 | 0.23 |
| | NC, ABPV | 1 | 0.23 |
| | NC, BQCV | 1 | 0.23 |
| 3 | NA, ABPV, DWV | 4 | 0.96 |
| | NA, BQCV, DWV | 2 | 0.46 |
| | NA, BQCV, SBV | 1 | 0.23 |
| | ABPV, BQCV, DWV | 4 | 0.96 |
| | ABPV, CBPV, DWV | 1 | 0.23 |
| | SBV, CBPV, DWV | 1 | 0.23 |
| 4 | NA, ABPV, BQCV, DWV | 2 | 0.46 |
| | NC, ABPV, BQCV, DWV | 1 | 0.23 |

For explanations see Table 2.

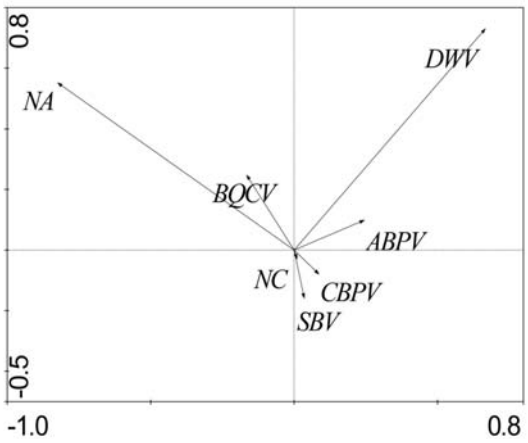


Fig. 1. PCA ordination diagram of virus coinfections in honeybees in the years 2006–2009. First two axes are displayed. The first axis accounts for 33.4% and the second axis 24.6% of the variability. NA –*Nosema apis;* NC –*Nosema ceranae;* PL –*Paenibacillus* larvae; DWV – Deformed wing virus; ABPV – Acute bee paralysis virus; SBV – Sacbrood virus; CBPV – Chronic bee paralysis virus; BQCV – Black queen cell virus.

geographical borders and infect new hosts. The *V. destructor* mite, which originally only infested *A. cerana,* but now infests *A. mellifera*, is a good example. Similarly, the transmission of *N. ceranae*, which naturally

Table 5. Results of chi-square test for all types of coinfections in honeybees.

| No. of viruses | Type of coinfection | Chi-square (d.f. = 1) | *P* level | Bonferroni correction $1–(1–p)^n$, |
|---|---|---|---|---|
| | | | | $\beta = 0.002$ |
| 2 | NA, NC | 1.73 | 0.19 | 0.99 |
| | NA, ABPV | 11.55 | **0.001** | 0.014 |
| | NA, BQCV | 14.25 | **0.00016** | **0.003** |
| | NA, SBV | 5.2 | **0.023** | 0.38 |
| | NA, CBPV | 4.75 | **0.029** | 0.46 |
| | NA, DWV | 19.17 | **0.00001** | **0.0003** |
| | NC, ABPV | 0.06 | 0.8 | 1 |
| | NC, BQCV | 1.92 | 0.17 | 0.98 |
| | NC, SBV | 0.67 | 0.41 | 1 |
| | NC, CBPV | 0.4 | 0.53 | 1 |
| | NC, DWV | 0.52 | 0.47 | 1 |
| | ABPV, BQCV | 1.13 | 0.29 | 0.999 |
| | ABPV, SBV | 1.09 | 0.3 | 0.999 |
| | ABPV, CBPV | 1.9 | 0.17 | 0.98 |
| | ABPV, DWV | 2.41 | 0.12 | 0.93 |
| | BQCV, SBV | 0.11 | 0.75 | 1 |
| | BQCV, CBPV | 0.91 | 0.34 | 1 |
| | BQCV, DWV | 0.01 | 0.91 | 1 |
| | SBV, CBPV | 1.3 | 0.25 | 0.998 |
| | SBV, DWV | 1.4 | 0.24 | 0.997 |
| | CBPV, DWV | 0.22 | 0.64 | 1 |
| | | | $\beta = 0.008$ | |
| 3 | NA, ABPV, DWV | 38.72 | $< \mathbf{10^{-6}}$ | $< \mathbf{10^{-6}}$ |
| | NA, BQCV, DWV | 34.32 | $< \mathbf{10^{-6}}$ | $< \mathbf{10^{-6}}$ |
| | NA, BQCV, SBV | 21.1 | $< \mathbf{10^{-5}}$ | $< \mathbf{10^{-4}}$ |
| | ABPV, BQCV, DWV | 36.58 | $< \mathbf{10^{-6}}$ | $< \mathbf{10^{-6}}$ |
| | ABPV, CBPV, DWV | 4.52 | **0.033** | 0.185 |
| | SBV, CBPV, DWV | 11.91 | $< \mathbf{10^{-3}}$ | **0.0033** |
| | | | | $\beta = 0.025$ |
| 4 | NA, ABPV, BQCV, DWV | 77.4 | $< \mathbf{10^{-6}}$ | $< \mathbf{10^{-6}}$ |
| | NC, ABPV, BQCV, DWV | 75.69 | $< \mathbf{10^{-6}}$ | $< \mathbf{10^{-6}}$ |

For explanations see Table 2.

infects *A. cerana*, was discovered a decade ago infecting *A. mellifera* and now is a threat to apiaries (Fries et al. 2006).

This study focused on the co-existence, occurrence, prevalence and distribution of six viruses, two types of nosemosis disease and American foulbrood. The analysis of ailing bee colonies provides a very interesting picture of their pathological state, but statistically this survey highlights only the number of apiaries threatened by disease or individual bees infected. This study does not provide information on the percentage representation of individual diseases at a particular locality, for this both healthy and ill bee colonies in an area would have to be sampled. Only SBV and CBPV of the viruses screened show distinguishable clinical symptoms, which can easily be recognized by a beekeeper, while other viruses can persist in bee colonies and cause so-called non-apparent infections. These asymptomatic infections can remain hidden as bees or bee colonies with these infections are not currently sampled and sent for diagnosis. Because the relationship of these infections to the mortality experienced by bee colonies is unknown it would be useful to obtain information on the early development of these non-apparent infections.

The results record the presence of particular bee viruses, nosemosis and American foulbrood in samples collected from ailing bee colonies between 2006 and 2009. During this period, the number of coinfections increased significantly. Coinfections with two agents consisted of infections with nosemosis and a virus, or two viruses. A similar increase in multiple infections, triple and quadruple coinfections was also recorded between 2006 and 2009. As these records are for dead bees and larvae from ailing or collapsing bee colonies, they are for bees whose immune system was unable to protect them. The number of infections and coinfections increased between 2006 and 2009. Also, the number of cases of infection with DWV, ABPV and BQCV increased. As the bees that did not show any symptoms of infection were not sampled it is not possible to state how many bee colonies were infected but were able to cope with the infection and did not show any clinical symptoms. However, it is likely that the movement of colonies of bees globally initiated an increase in infestation of colonies by mites and infections with pathogens, which resulted in the death of susceptible bee colonies over winter, leaving only those that were able to cope with the mites and pathogens (VanEngelsdorp & Meixner 2010).

The chi-square tests indicate that the viruses often tend to occur simultaneously. i.e., a bee is often infected with two or more viruses, which is especially true for the combination BQCV and DWV, and of *N. apis* with each of the viruses ABPV, SBV and CBPV. The incidence of multiple infections has increased recently. Similarly, the CANOCO analysis indicates that BQCV occurs mainly in coinfections with *N. apis* and DWV virus with ABPV virus. To summarize, coinfections with these pathogens are more common than expected if they are assumed to be distributed at random in the bee population.

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