# First molecular confirmation of deformed wing virus infections of honeybees from a Belgian apiary reveals the presence of black queen cell virus and *Varroa destructor* virus 1

*De eerste moleculaire bevestiging van deformed wing virus infecties van honingbijen van een Belgische bijenstand onthult de aanwezigheid van black queen cell virus en* Varroa destructor *virus 1*

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## ABSTRACT

**Eight emerging young bees with wing deformities were examined using existing RT-PCR tests for the presence of viral agents. All samples tested positive for deformed wing virus and *Varroa destructor* virus 1. Six out of eight bees also tested positive for black queen cell virus (BQCV). This is the first report that confirms the presence of honeybee viruses in Belgium by molecular means. The finding of BQCV is remarkable, as no typical signs of this disease have ever previously been reported in our country.**

## SAMENVATTING

Acht ontluikende jonge bijen met vleugelmisvormingen werden onderzocht met behulp van bestaande RT-PCR-testen op de aanwezigheid van virale agentia. Alle stalen testten positief voor deformed wing virus en *Varroa destructor* virus 1. Zes van de acht bijen testten eveneens positief voor black queen cell virus (BQCV). Dit is de eerste verslaggeving waarin op moleculaire wijze de aanwezigheid van bijenvirussen in België wordt bevestigd. Het vinden van BQCV is opmerkelijk daar de typische tekenen van deze ziekte nog niet werden beschreven in ons land.

## INTRODUCTION

The economic value of honeybees in Belgium is estimated to be 472 million dollars, nearly all of which relates to their contribution as pollinators (Simoens *et al.*, 2003). This places bees at position four – just below cattle, pigs and poultry – in the ranking of most important "farm" animals. However, worldwide the domesticated European honeybee *Apis mellifera* is increasingly being threatened by infectious agents, including parasites, bacteria, fungi and viruses. Although the reports about viral diseases have long been sporadic, even anecdotic in Belgium, this changed dramatically in 1984 when the bee parasitic *Varroa* mite was introduced into our country. In the early 1960s, *Varroa destructor* (previously mislabeled as *Varroa jacobsoni*), originally confined to the Asian honeybee *Apis cerana*, jumped to the *mellifera* bees, which were completely defenseless against this new ectoparasite. In addition, the hematophagous mite caused the easy transmission of several bee viruses by puncturing their mouthparts through the insects' larval or pupal skin during the blood meal. It has been suggested that the mite can also trigger replication of viruses already present in bees by the simple mechanical effect of cuticle piercing or by injection of external proteins into the insect hemolymph (Colin *et al.*, 2002). In fact, these viruses now seem to cause much more damage to the developing honeybee brood than the losses of essential nourishment caused by the feeding mite. Some of the clinical symptoms previously attributed to high *Varroa* loads have been proven to be caused by the transmitted viruses: i) deformities to the wings of emerging bees are caused by the deformed wing virus (DWV) (Bowen-Walker *et al.*, 1999) and ii) the acute bee paralysis virus (ABPV) has been identified as a major factor contributing to the mortality of honeybees in colonies infested by *Varroa* (Ball and Allen, 1988). In addition, the putative role of the *Varroa* mite in the transmission of sacbrood virus (SBV) and kashmir bee virus (KBV) has been supported by the findings of the corresponding virus RNA in *Varroa* mites (Tentcheva *et al.*, 2004a). However, bee viruses can also be transmitted by other means: ABPV also spreads by way of the salivary gland secretions of the adult bees and in

**Table 1. PCR primer sets for the identification of different target viruses.**

| Virus | Orient. | Sequence | Amplicon length (bp) | Ref.* |
|---|---|---|---|---|
| DWV | Fwd | 5'-GTAAGCGTCGTGAACATACTG-3' | | |
| | Rev | 5'-GACTCTCTCCCGCGAGA-3' | 1129 | [1] |
| BQCV | Fwd | 5'-TGGTCAGCTCCCACTACCTTAAAC-3' | | |
| | Rev | 5'-GCAACAAGAAGAAACGTAAACCAC-3' | 700 | [2] |
| ABPV | Fwd | 5'-TTATGTGTCCAGAGACTGTATCCA-3' | | |
| | Rev | 5'-GCTCCTATTGCTCGGTTTTTCGGT-3' | 900 | [2] |
| SBV | Fwd | 5'-ACCAACCGATTCCTCAGTAG-3' | | |
| | Rev | 5'-CCTTGGAACTCTGCTGTGTA-3' | 487 | [3] |
| CBPV | Fwd | 5'-AGTTGTCATGGTTAACAGGATACGAG-3' | | |
| | Rev | 5'-TCTAATCTTAGCACGAAAGCCGAG-3' | 455 | [4] |
| KBV | Fwd | 5'-GATGAACGTCGACCTATTGA-3' | | |
| | Rev | 5'-TGTGGGTTGGCTATGAGTCA-3' | 393 | [5] |
| VDV-1 | Fwd | 5'-CGAAACGAAGAGAGCATGTAT-3' | | |
| | Rev | 5'-CGACTCTTCCCCAGCTAAG-3' | 1129 | [1] |

* [1] Ongus *et al.*, 2004; [2] Benjeddou *et al.*, 2001; [3] Grabensteiner *et al.*, 2001; [4] Ribière *et al.*, 2002; [5] Stoltz *et al.*, 1995.

food stores to which these secretions are added (Ball, 1985). And recently, the vertical transmission of viruses from honeybee queens to their offspring has been suggested; DWV, SBV, KBV, chronic bee paralysis virus (CBPV) and black queen cell virus (BQCV) were involved (Chen *et al.*, 2006a).

Up to the late 1990s, the diagnosis of viral bee diseases was based on immunoassays, with the immunodiffusion test being the most commonly used (Ribière *et al.*, 2000). However, an important drawback of these antigen detection assays was the requirement of virus-specific hyperimmune serum (mostly from rabbits), which was not commercially available. In addition, the production of this serum was hampered by the near inability of the manufacturers to make pure preparations of a single virus species, due to fact that most bees are infected by multiple viruses with similar physical characteristics. In the past decades, reverse transcriptase-polymerase chain reaction (RT-PCR; Stoltz *et al.*, 1995; Benjeddou *et al.*, 2001; Grabensteiner *et al.*, 2001; Ribière *et al.*, 2002; Ongus *et al.*, 2004; Tentcheva *et al.*, 2004b; Genersch, 2005), multiplex RT-PCR (Topley *et al.*, 2005) and quantitative RT-PCR (qRT-PCR; Chen *et al.*, 2005; Tentcheva *et al.*, 2006; Ward *et al.*, 2007) techniques have been developed for several of these picorna-like honeybee viruses, making the diagnosis better accessible for laboratories that have the equipment for molecular work. Although historically there are many convincing descriptions of the clinical signs of viral diseases made by Belgian beekeepers and scientists, the affirmative data is still lacking. Bees with wing deformities are very common in Belgian apiaries with high levels of *Varroa* infestation, and clusters of flightless, trembling, crawling bees, sometimes almost hairless, are likewise often seen here. They are most probably indicative of DWV and CBPV infections, respectively. In this paper we present the first molecular confirmation of the presence of bee viruses in a Belgian apiary. A sample of eight adult bees suspected of having DWV infection was tested with existing RT-PCR procedures for a broad range of bee viruses, including DWV, BQCV, ABPV, SBV, CBPV, KBV, and one mite virus, *Varroa destructor* virus 1 (VDV-1).

## MATERIALS AND METHODS

### Sample collection and photography

Sample collection was done at the apiary in the Laboratory of Zoophysiology on the "Sterre" campus of Ghent University. Eight emerging young bees with wing deformities were taken from a sealed brood comb that had been placed in an incubator at 34°C. Each sampled bee was immediately thereafter placed individually in a microtube and killed by freezing. Just before RNA preparation, photographs were made of the thawed and air-dried bees under a stereoscope (Olympus type CXZ-9 equipped with a Colorview 8 digital camera).

### RNA isolation

Prior to homogenization by grinding in a microtube using a teflon pistle, the entire digestive tube of each bee was removed by dissection. All remaining tissues were then put in 500 µl phosphate buffered saline (PBS, pH 7.2) and homogenized at room temperature. RNA preparations of each bee individually were made using the QIAamp Viral RNA Mini Kit (Qiagen) according to the "spin protocol". Viral RNA was stored at –20°C.

### Reverse transcriptase PCR

cDNA was made using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) with oligo (dT)18 primers according to the manufacturer's instructions. Thereafter, for each sample seven PCR

**Table 2. BLASTN search of the nucleotide sequences of the detected amplicons.**

| Amplicon | ID* | Acc. number | Score | Coverage (%) | E-value | Identity (%) |
|---|---|---|---|---|---|---|
| DWV_8 | DWV | AJ489744 | 1825 | 99 | 0.0 | 97 |
| | DWV | AY292384 | 1792 | 99 | 0.0 | 97 |
| | DWV | AY224602 | 1759 | 99 | 0.0 | 96 |
| BQCV_8 | BQCV | AY626246 | 1190 | 100 | 0.0 | 98 |
| | BQCV | AF183905 | 1157 | 100 | 0.0 | 97 |
| | BQCV | DQ364629 | 1050 | 93 | 0.0 | 96 |
| VDV1_8 | VDV-1 | AY251269 | 1905 | 99 | 0.0 | 99 |
| | DWV | AJ489744 | 1024 | 99 | 0.0 | 84 |
| | DWV | AY224602 | 1014 | 99 | 0.0 | 84 |

* Only the 3 best matches are given.

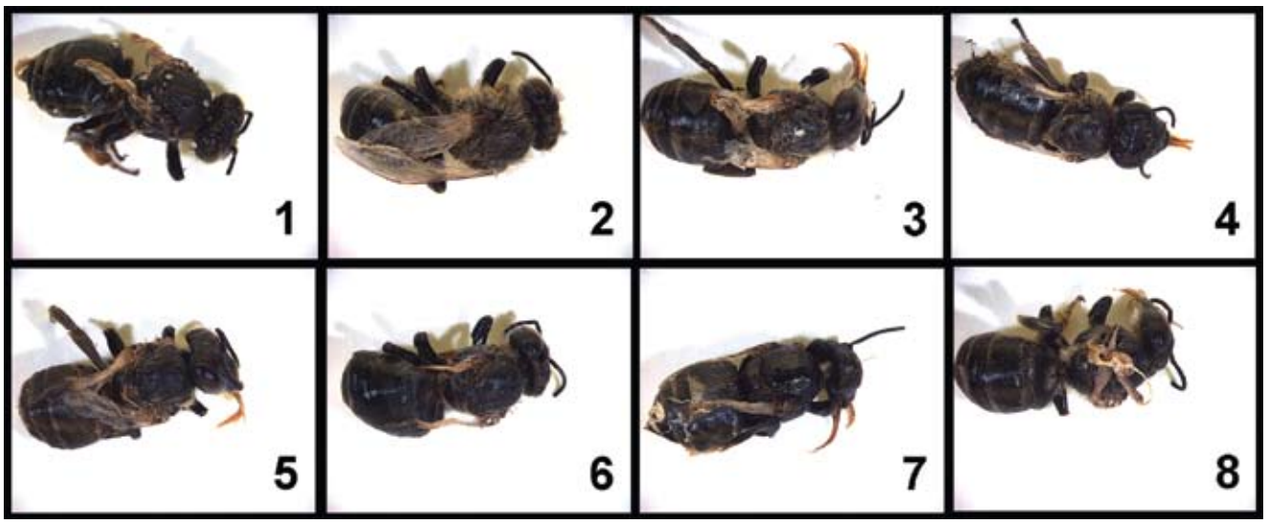


**Figure 1. The eight emerging young bees examined had the typical signs of DWV infection with rudimentarily developed or shrunken wings, a shortened abdomen and discoloring.**

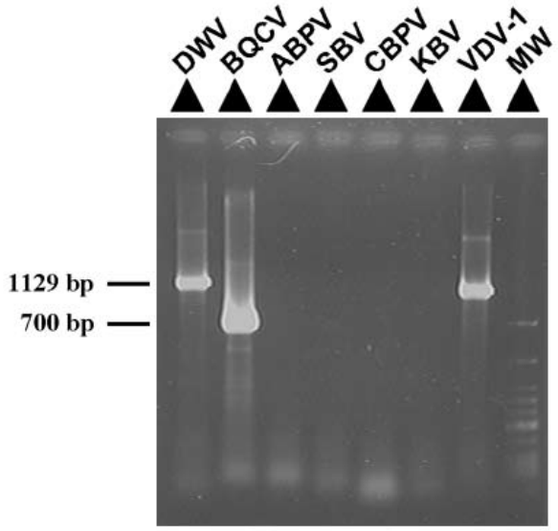


**Figure 2. Results of the RT-PCR tests of sample 7. Positive outcomes were obtained with DWV, BQCV and VDV-1, resulting in an amplicon of 1129, 700 and 1129 bp, respectively. The other viruses (ABPV, SBV, CBPV and KBV) tested negative. Abbreviations of the bee viruses are explained in the text. MW = molecular weight marker.**

reactions were set up following the same protocol, but only differing in the chosen target virus-specific primer set (Table 1). PCR assays were done as follows: 1 µl of cDNA was mixed with 2.5 µl of 10x PCR buffer, 5 µl Q-solution, 1 µl of a solution containing each deoxynucleoside triphosphate at a concentration of 10 mM, each primer at a concentration of 2 µM, and 1.25 U HotStarTaq DNA polymerase (Qiagen); the final volume of the mixture was 25 µl. The mixtures were heated in an Eppendorf Mastercycler for 15 min at 95°C, and this was followed by 31 amplification cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) and then by 7 min at 72°C to complete the polymerization. The PCR products were analyzed by 2% agarose gel electrophoresis.

## DNA sequencing

DNA sequencing was performed using a Perkin Elmer ABI Prism 377 automated DNA sequencer. PCR products were first purified using the Illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare) and then treated with shrimp alkaline phosphatase (1 U/µl, Amersham) and exonuclease I (20 U/µl, Epicentre Technologies) for 15 minutes at

37°C, followed by 15 minutes at 80°C to inactivate the enzymes. This material was then used for cycle sequencing without any further purification, using the ABI Prism BigDye V 3.1 Terminator Cycle Sequencing kit. The sequencing conditions were 30 sec at 96°C, 15 sec at 50°C and 4 min at 60°C for 27 cycles. Both the forward and reverse primers of each target virus were used for sequencing. Cycle sequence products were precipitated by adding 25 µl of 95% ethanol and 1 µl 3 M sodium acetate, pH 4.6 to each cycle sequencing reaction (10 µl). The samples were placed at –20°C for 15 minutes and centrifuged at 14.000 rpm for 15 minutes. After precipitation, an additional wash of the pellet was performed with 125 µl of 70% ethanol and centrifuged at 14.000 rpm for 5 minutes. The pellet was dried in a Speedvac concentrator, resolved in loading buffer and run on a 48 cm 4.25% acrylamide:bisacrylamide (29:1) gel.

The resulting DNA sequences were BLAST-searched on the world wide web (http://www.ncbi.nlm.nih.gov/BLAST/).

## RESULTS

As demonstrated in Figure 1, all bees examined had the typical signs of DWV infection with rudimentary developed or shrunken wings, a shortened abdomen and discoloring (Genersch, 2005). All samples tested positive with RT-PCR for DWV and VDV-1 (Figure 2). Six out of eight bees also tested positive for BQCV (bees 2, 3, 5-8). The corresponding PCR products from sample (bee) 8 were purified and sequenced. BLAST-searching revealed that the found sequences correspond to the DWV gene for polyprotein (AJ489744), the BQCV structural polyprotein gene (AY626246) and the genome sequence of VDV-1 (AY251269) (Table 2).

## DISCUSSION

This is the first molecular confirmation of the presence of DWV, BQCV and VDV-1 in honeybee workers in Belgium. All three viruses are positive sense single-stranded RNA viruses belonging to the picorna-like virus superfamily and their complete genome sequences are available (Leat *et al.*, 2000; Ongus *et al.*, 2004). Based on their genomic organization, BQCV has been assigned to a new virus family named *Dicistroviridae*, whereas DWV and VDV-1 are assigned to the genus *Iflavirus*, which is a "floating genus" that has not yet been assigned to a family (from Chen *et al.*, 2006b). DWV and BQCV are typical honeybee viruses causing different pathologies, whereas VDV-1 was originally isolated from the *V. destructor* mite in which it replicates. The biological properties of VDV-1 in bees are not yet known. VDV-1, DWV and also KBV are closely related, suggesting that they have evolved from a common ancestor (Ongus *et al.*, 2004). The sequence similarity between VDV-1 and DWV was also demonstrated by our BLASTN-search with the VDV-1 amplicon, where the second and third best hits were DWV sequences.

DWV is one of the most common honeybee viruses (Tentcheva *et al.*, 2004a). It is thought to be responsible for wing deformities when infection occurs during the white-eyed pupal stage (Bailey and Ball, 1991), but the mechanisms behind these symptoms are not clearly understood. The typical injuries on the wings of the workers mostly occur in those heavily infested by *V. destructor*. The mite certainly is an important actor in the spread of this virus, but transmission by food secretions from nurse bees to larvae and from queen to workers has also been demonstrated (Chen *et al.*, 2006a). Although the clinical signs of highly infested bees are very pronounced, in general DWV might be considered poorly pathogenic (Bowen-Walker *et al.*, 1999; Tentcheva *et al.*, 2004b).

BQCV was originally found in dead honeybee queen larvae and pupae (Bailey and Woods, 1977). The name of the virus was derived from the darkened areas on the walls of the cells containing infected pupae. To our knowledge, no such signs of disease have ever previously been reported in Belgium. BQCV was found to be the most common cause of death of queen larvae in Australia (Anderson, 1993). In an epidemiological study in France, this virus was very prevalent, especially in adult bees, whereas infections in pupae were scarcely detected (Tentcheva, 2004a). There seems to be a close association between BQCV and *Nosema apis*, a microsporidian midgut parasite of adult bees. Per os administration of the virus was shown to be totally dependent on the presence of the parasite (Bailey *et al.*, 1983). With the emergence of the more pathogenic *Nosema ceranae* in apiaries of the Western honeybee (Higes *et al.*, 2006), the finding of BQCV here in Belgium could be of significance. It would be interesting to investigate the involvement of concurrent infections of BQCV and *N. ceranae* in the context of the colony collapse disorder, a little-understood worldwide phenomenon in which worker bees from Western honeybee colonies abruptly disappear.

The present paper proves that different honeybee viruses are circulating in Belgian apiaries. The finding of the DWV genome is in agreement with the typical signs of the worker bees examined. However, the molecular confirmation of the presence of BQCV and VDV-1 does not necessarily have any clinical importance. Recently it has been demonstrated that a correlation exists between high CBPV genomic load and pathology expression (Blanchard *et al.*, 2007). Therefore, the virus infections in bee colonies should preferentially be monitored in a quantitative way and threshold values representing dangerously high virus loads should be set. Only then can tests for identification and quantification of honeybee viruses be offered as a service for the beekeepers. The observation that BQCV virus occurs in Belgium is valuable as it helps us to choose which virus to focus on.

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