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Incidence and molecular characterization of viruses found in dying New Zealand honey bee (*Apis mellifera*) colonies infested with *Varroa destructor**

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Abstract – The virus status of New Zealand honey bee colonies infested with *Varroa destructor* was studied from 2001 to 2003. The viruses CBPV, BQCV, SBV, CWV, and KBV were all found during the study, with CWV and KBV the most common, as inferred from serological and protein profile analyses. DWV, SPV and ABPV were not detected in these colonies. CWV was present in the colonies throughout the season, while the appearance of KBV generally coincided with autumn colony collapse when *V. destructor* populations were large. Inconsistencies between serological analyses and viral capsid protein profiles of the extracts containing CWV and KBV were probably a result of strain differences between the viruses found in New Zealand and those used to generate the diagnostic antisera. The genome of the New Zealand KBV strain was partially sequenced. Phylogenetic and serological analyses showed this strain to be unique and most closely related to Canadian KBV isolates.

Apis mellifera / Kashmir bee virus / *Varroa destructor* / New Zealand

1. INTRODUCTION

Varroa destructor Anderson & Trueman was first detected in New Zealand in 2000 (Zhang, 2000). Since then, the number of managed *Apis mellifera* L. colonies has decreased by at least 16% in the country's North Island where the mites have become established (Goodwin, 2004a). Studies of colony losses in other countries where *V. destructor* is present have suggested a link between colony mortality and the presence of overt viral infections in the infested bee population (Ball and Allen, 1988; Ball, 1997; Martin et al., 1998; Nordström et al., 1999; Tentcheva et al., 2004b). Furthermore, several independent studies have shown that adult female mites acquire viruses from infected bees and

transmit them to healthy adult bees or pupae on which the mites subsequently feed (Allen et al., 1986; Bowen-Walker et al., 1999; Brødsgaard et al., 2000; Nordström, 2003; Chen et al., 2004). Infection with virus during the bees' larval or pupal stages can result in death of pupae or reduced longevity or deformation of newly emerged adults, depending on the type of virus transmitted and the amount of virus replication (Bowen-Walker et al., 1999; Brødsgaard et al., 2000; Martin, 2001; Tentcheva et al., 2004a; Chen et al., 2005). A decline in bee population resulting from the death of virus-infected brood and adults contributes to the collapse of mite-infested colonies, an outcome that is predicted by simulation models of mite, bee and virus population dynamics (Martin, 2001; Sumpter and Martin, 2004).

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Given that viruses transmitted by *V. destructor* are a significant factor in the collapse and death of *A. mellifera* colonies, it is likely that some colony losses that have occurred in New Zealand since the arrival of *V. destructor* have been partly due to the viruses transmitted by the mites. Prior to the arrival of *V. destructor*, ten honey bee viruses had been detected in New Zealand honey bees (Anderson, 1985, 1988; Allen and Ball, 1996) including Kashmir bee virus (KBV) and acute bee paralysis virus (ABPV). Both of these viruses have been detected as overt fatal infections in mite-infested colonies in other countries (Ball and Allen, 1988; Hung et al., 1996; Bakonyi et al., 2002). KBV, which normally exists as harmless inapparent infections of adult and pupal honey bees (Anderson, 1991), but is the most lethal of honey bee viruses when artificially injected into live honey bees (Dall, 1985), has long been predicted as a possible cause of bee mortality if it was transmitted by *V. destructor* (Anderson, 1991; Bailey and Ball, 1991). This is suspected to have happened in the USA (Hung et al., 1996; Chen et al., 2004; Shen et al., 2005), and recent studies have detected the virus in both bees and mites in infested colonies in Europe (Tentcheva et al., 2004b; Siede et al., 2005). Like KBV, ABPV exists as a harmless inapparent infection of honey bees, but becomes rapidly lethal when artificially injected into the haemolymph of adult bees and pupae (Bailey, 1965). Simulation models for the transmission of this virus (Martin, 2001; Sumpter and Martin, 2004) suggest that ABPV will only cause colony death when the mite population is large ($\geq 10\,000$ mites) since the rapid death of ABPV-infected bees prevents smaller mite populations from acquiring and spreading the virus. It is possible that this scenario is also true for colonies infected with KBV.

Two other viruses that have been associated with the collapse of colonies infested with *V. destructor* are slow paralysis virus (SPV) and deformed wing virus (DWV) (Ball, 1997; Martin et al., 1998; Nordström et al., 1999). To date, neither of these viruses has been detected in New Zealand honey bees. Unlike KBV and ABPV, DWV is not a rapidly fatal infection (Bowen-Walker et al., 1999; Nordström,

2003). Most DWV infected pupae survive until emergence, allowing the mites to complete their reproduction in the pupal phase and acquire virus in the process. Although adult bees with symptomatic DWV infections have a severely reduced lifespan of only a few days, the mites they carry will have had enough time to feed on them and acquire the virus. Moreover, asymptomatic bees from the same colonies, with greater longevity, can also carry large DWV titres (Bowen-Walker et al., 1999; Nordström, 2003; Tentcheva et al., 2004a; Chen et al., 2005; Yue and Genersch, 2005; Lanzi et al., 2006) thus providing a more long-lived source of inoculum. Consequently, it is possible a DWV epidemic within a colony can be sustained with a much smaller mite population (3000–4000 mites/colony) compared to more virulent viruses (Sumpter and Martin, 2004).

Since 2000, beekeepers in the North Island of New Zealand have increasingly observed dead adult bees and brood in colonies severely infested with *V. destructor* (Goodwin, 2004b). Research elsewhere has shown that such colony collapse may be in part due to epidemic viral infections, with *V. destructor* a possible vector for some of these (Ball and Allen, 1988; Bowen-Walker et al., 1999; Nordström et al., 1999; Brødsgaard et al., 2000; Tentcheva et al., 2004b). Consequently, we undertook a study to determine which honey bee viruses were present in collapsing mite-infested colonies in New Zealand and whether there were any seasonal changes in viral prevalence in the colonies. Such information could help to improve control strategies by determining the optimum time to apply acaricide treatments, both to keep the mite population below the critical level for transmission of the damaging viruses, and to help prevent any seasonal increases in these viruses in New Zealand colonies.

2. MATERIALS AND METHODS

2.1. Study colonies and sample collection

In 2001, 30 honey bee colonies were established at two apiary sites in the Bay of Plenty region of

New Zealand. These colonies were managed without acaricide treatment under regular observation until they died in 2002. Samples of dead bees, when they were present, were collected from dead bee traps placed at the entrance of each colony between November 2001 and May 2002, by which time all of the colonies had died. A sample of live bees was also collected from each of the colonies that were alive in March but later died (11 colonies) and again from the 12 remaining colonies in April 2002.

In November 2002, 13 small honey bee colonies were established at the Mt Albert Research Centre in Auckland, New Zealand. These colonies were managed without acaricide treatment for the duration of the study. The mean population of mites in each colony was estimated monthly from counts of mites obtained from a sub-sample of 500 adult bees according to Goodwin and Van Eaton (2001) and the data were analysed with an analysis of variance (ANOVA). Samples of dead bees, when present, were obtained from each of the 13 colonies on a monthly basis until the death of each colony during 2003.

The dead bee samples were analysed by serology and protein profiling (see below) for the presence of eight different honey bee viruses: black queen cell virus (BQCV), sacbrood virus (SBV), slow paralysis virus (SPV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), deformed wing virus (DWV) and cloudy wing virus (CWV). The samples of live bees that had been collected just prior to the death of the colonies in March and April 2002 (i.e., samples from 11 colonies that subsequently died in March and 12 that died in April 2002) were similarly analysed for these viruses. In addition, these live bee samples were analysed by infectivity tests in adult bees and pupae, followed by serological and protein profile analyses of the inoculated individuals.

2.2. Bee extracts for virus detection

In order to determine which bee viruses were present in these colonies when sampled, and in what amounts, a composite virus purification protocol reflecting the different purification requirements for CWV and DWV on the one hand, and those of BQCV, SBV, SPV, CBPV, ABPV and KBV on the other (Bailey and Ball, 1991) was used to prepare mixed extracts from live and dead bees for serology and protein profile analysis. Briefly, 30 bees were homogenised in 30 mL 0.01 M potas-

sium phosphate buffer (PB) pH 6.7 with 0.02% diethyldithiocarbamic acid (DIECA) and 3 mL carbon tetrachloride (CCl_4). The homogenate was filtered through cotton wool and centrifuged at 12 000 *g* for 20 min, retaining both the pellet ('P') and supernatant ('S') fractions for analysis. At this molarity and pH, CWV and DWV tend to aggregate and precipitate, while the other viruses remain suspended in the 'S' fraction. The 'S' fraction was centrifuged at 75 000 *g* for 3 h, producing a pellet that was resuspended in 1 mL 0.01 M PB (pH 6.7), while the 'P' fraction was resuspended in 1 mL 0.2 M PB (pH 8.0). Both suspended fractions were then centrifuged at 8000 *g* for 10 min to remove particulate matter and the supernatants retained.

2.3. Serological analysis

The 'S' extracts (suitable for the serological detection of BQCV, SBV, SPV, CBPV, ABPV and KBV) and 'P' extracts (for DWV and CWV) were tested against specific antisera to these viruses by agar-gel immunodiffusion (AGID), as described by Bailey and Ball (1991). Each antiserum was used at a working strength concentration that was 4× more concentrated than the end-point titre for detecting the respective virus. Because of the serological variability of KBV (Allen and Ball, 1995), several different strain-specific antisera (KBV-NewZealand, KBV-SouthAustralia, KBV-India, KBV-Canada, KBV-Spain, KBV-NZ1, KBV-NZ2, KBV-NZ4 and KBV-NZ6) were employed for detecting this virus. All antisera except KBV-NZ1, KBV-NZ2, KBV-NZ4 and KBV-NZ6 were produced in rabbits at Rothamsted Research, UK, against highly purified virus preparations using standard protocols and were devoid of antibodies against contaminating viruses or host proteins (Harlow and Lane, 1988). Each of these antisera was used at a higher concentration (i.e., 6× or 8× more concentrated than the endpoint titre) to increase the probability of detecting a New Zealand virus strain which differed from those used to produce the antiserum. The remaining four antisera were a gift from Dr D.L. Anderson and were produced in rabbits against different New Zealand isolates of KBV (Anderson, 1985) and were used at 4× the end-point titre. The 'P' extracts obtained from the dead bee samples were also tested for DWV and CWV by an indirect double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) adapted from Allen et al. (1986), which is considerably more sensitive than AGID.

2.4. Protein profile analyses

The 'S' extracts prepared from each of the dead and live bee samples were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Allen and Ball (1995). The sizes of the viral capsid proteins were calculated by reference to commercial molecular weight standards and compared with the protein profiles of known honey bee viruses (Bailey et al., 1979; Bailey and Ball, 1991; Allen and Ball, 1995).

2.5. Infectivity tests

Infectivity tests, as described by Bailey and Woods (1977), were conducted on the live bee samples collected in March and April 2002. The heads from five live bees were homogenised in 0.5 mL 0.01 M PB pH 6.7 with 0.02% DIECA and 50 μ L CCl_4 and centrifuged at 8000 *g* for 10 min. Serial ten-fold dilutions of the supernatant were made in sterile 0.01 M PB pH 6.7. Twenty adult bees and 20 white-eyed pupae from mite-free colonies at Rothamsted Research, UK, were each injected with 1 μ L of (diluted) extract. The adult bees had been caged as newly emerged individuals and maintained in incubators for one week before injection. Following injection, the test bees were maintained in cages and the pupae in Petri dishes in incubators at 30 °C and checked daily for mortality. Control adult bees and pupae were injected with 1 μ L of buffer and maintained similarly. Bee virus extracts of individual dead bees and pupae from each dilution were prepared as described above for the live bee heads except that each bee was homogenised in 1 mL 0.01 M PB pH 6.7 with 0.02% DIECA and 0.1 mL CCl_4 . Each extract was tested by AGID using a range of antisera, and protein profiles of extracted viruses visualised using SDS-PAGE as described above.

2.6. Reverse Transcriptase-PCR analysis

Viral RNA was purified from the 2002–2003 virus extracts using the RNA cleanup protocol of the RNeasy™ RNA isolation kit (Qiagen) eluting into 30 μ L nuclease-free water. The RNA samples were screened for KBV, ABPV and DWV by semi-nested RT-PCR using primer sets 1, 2 and 6 (Tab. I). The KAPBV set of primers (primer set 1) were designed to reverse transcribe (KAPBV-REV2) and

amplify (KAPBV-FOR & KAPBV-REV1) a wide range of potential variants within the ABPV-KBV species complex (de Miranda et al., 2004). They are located in regions with high nucleotide identity across all publicly available ABPV and KBV sequences, with at most a single nucleotide mismatch between ABPV and KBV (underlined in each primer) and with the 3' end of each primer coinciding with the 1st codon position of its corresponding amino acid. Primer KBV-FOR is located in a region where there is a gap in the ABPV-KBV alignments (δ), as well as many mismatches (underlined), and is used together with KAPBV-REV1/KAPBV-REV2 for amplifying only KBV variants (primer set 2). The DWV primers for semi-nested RT-PCR (primer set 6) were derived in a similar manner from DWV alignments.

10 μ L of RNA was reverse transcribed with MuMLV (Promega) in a 50 μ L total volume containing the manufacturer's buffer, 1 μ M of the appropriate cDNA primer (Tab. I), and 500 μ M dNTP, for 30 min at 37 °C. 10 μ L of the resultant cDNA was amplified in 50 μ L total volume containing 1.5 μ M each of the corresponding PCR primers (Tab. I), 200 μ M dNTP, Taq polymerase (Promega) and the manufacturer's buffer, and the following incubation schedule: 94 °C: 2 min + 30 \times [94 °C: 30 s – 59 °C: 30 s – 72 °C: 30 s] + 72 °C: 2 min. The annealing temperature, incubation times and primer concentrations were optimised for the sensitivity of detection of these viruses. The RT-PCR products were sequenced both directly and after cloning into pGEM-TEasy™ (Promega).

2.7. Cloning and sequencing the KBV-NZ genome

Bees and mites were collected from a severely infested colony in Auckland, New Zealand, in April 2004. The mites were crushed directly onto Whatman FTA® databasing paper and left to dry at room temperature, while the bees were frozen at –20 °C overnight before similarly being crushed and dried onto the FTA® paper. This paper is impregnated with a mix of chemicals that lyse cells, prevent bacterial growth and protect DNA and RNA from degradation. The FTA® cards were sent to Pennsylvania by mail and stored at room temperature for 1 month before use. Cores of the dried samples taken from the FTA® cards were soaked for 15 min in the RLT buffer of the RNeasy™ RNA isolation kit (Qiagen) followed by manufacturer's protocol for RNA cleanup, finally eluting

Table I. List of primer sets used for semi-nested and regular RT-PCR for the detection, cloning and sequencing of KBV, ABPV and DWV. Primer sets 1–5 are for amplifying both ABPV and KBV (sets 1 & 3) or only KBV (sets 2, 4, 5) while sets 6 & 7 are designed to detect DWV. For semi-nested RT-PCR, the ‘REV2’ extension denotes the cDNA primer and the ‘FOR’ and ‘REV1’ extensions the PCR primers. Underlined nucleotides mark positions where there are nucleotide differences between the KBV and ABPV sequences while the (δ) in primer KBV-FOR marks a gap in the KBV-ABPV alignment. The numbers refer to the positions of the corresponding nucleotides on the KBV and DWV genomes (de Miranda et al., 2004; Lanzi et al., 2006; respectively) and the nucleotides in lower case mark restriction enzyme recognition sites added to the 5' end of the primers.

Set	Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
1	KABPV-FOR	ggatc ⁷³³⁹ CAGTCTATATGTGGT ⁷³⁵³	KABPV-REV1	aagct ⁷⁸²⁰ TCCAGGCACATTCTG ⁷⁸⁰⁶
			KABPV-REV2	⁷⁸⁸⁹ TAATTCGTTATTGGGGTC ⁷⁸⁷²
2	KBV-FOR	gggatcc ⁷⁵⁵¹ GT(δ)TTCTATGCAAAATCGCA ⁷⁵⁶⁸	KABPV-REV1	aagct ⁷⁸²⁰ TCCAGGCACATTCTG ⁷⁸⁰⁶
			KABPV-REV2	⁷⁸⁸⁹ TAATTCGTTATTGGGGTC ⁷⁸⁷²
3	KABPV-Idr-FOR	ggg ⁶⁵⁴⁶ ATCCTCTATTGGATA ⁶⁵⁶⁰	KABPV-REV1	aagct ⁷⁸²⁰ TCCAGGCACATTCTG ⁷⁸⁰⁶
4	KBV-D-FOR	⁶⁰⁴⁹ CTTATACAGATGAGCTCAA ⁶⁰⁶⁷	KABPV-Idr-REV	aaagct ⁶⁷⁶⁵ TGGGGGTATCGATCC ⁶⁷⁵¹
5	KBV-I-FOR	³¹⁷¹ CTCGCTTTTGTACTTGCC ³¹⁸⁹	KBV-E-REV	⁴²⁹⁶ CITTCATGGTACTCTAAATAC ⁴²⁷⁵
6	DWV-FOR	ggat ⁷⁴⁵⁵ CCTAGAATCCATAGATTG ⁷⁴⁷²	DWV-REV1	aagct ⁷⁹⁴¹ TATGGTCGCCAGTTAC ⁷⁹²⁶
			DWV-REV2	⁷⁹⁶⁴ ATTCTCGAAAGCCAATAG ⁷⁹⁴⁷
7	DWV-J-FOR	⁶⁴⁵² TGCAGCTGGAATGAATGC ⁶⁴⁶⁹	DWV-K-REV	⁷⁹⁶⁴ ATTCTCGAAAGCCAATAG ⁷⁹⁴⁷

into 30 μ L nuclease-free water. These RNA samples were screened for KBV, ABPV and DWV using primer sets 1, 2 and 7 (Tab. I). The RNA was reverse transcribed and amplified as described above, except that the primer concentrations for PCR were 0.5 μ M of each primer and the cycling schedule was 35 cycles of 94 °C: 30 s – 55 °C: 30 s – 72 °C: 90 s.

The RT-PCR products were resolved on 1% agarose-TAE gels, excised, purified using the GelExtraction kit (Qiagen), and cloned into pGEM-TEasyTM (Promega). At least three independent clones were sequenced per ligation to obtain a consensus sequence representative of the principal sequence variant in the sample. The partial genome sequence of the New Zealand isolate of KBV thus obtained was extended with further RT-PCR amplifications employing six other primer pairs derived from the Pennsylvania KBV sequence (de Miranda et al., 2004). Only three amplifications (with primer sets 3–5; Tab. I) were successful. In all cases the RT-PCR products were cloned and three independent clones were sequenced.

2.8. Phylogenetic analyses

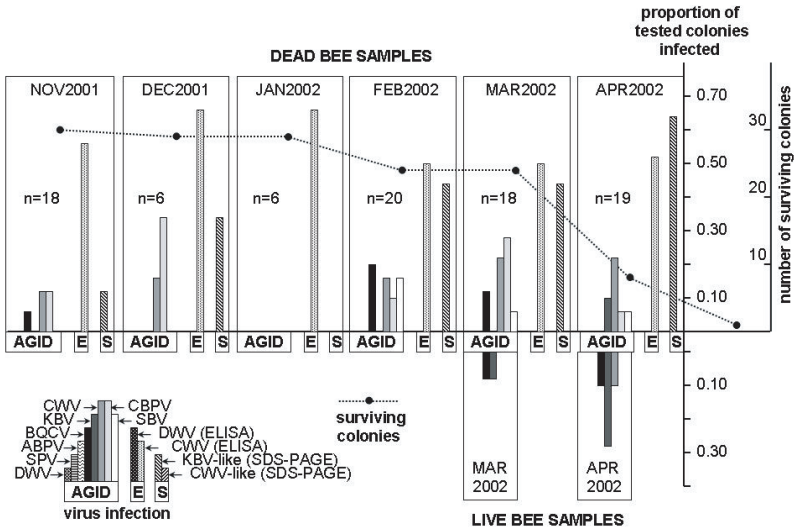
Multiple sequence alignments were constructed using CLUSTAL-W at <http://www.ebi.ac.uk/clustalw/> (Thompson et al., 1994) using *Solenopsis invicta* virus (Valles et al., 2004) as

the outgroup. A region covering 226 nucleotides (of which 77 were phylogenetically informative) of the VP4 gene was chosen for phylogenetic analysis, since this region allowed the inclusion of the greatest number of taxa. Phylogenetic inference followed maximum parsimony criteria, as implemented by PAUP4.0b10 (Swofford, 1998), with gaps treated as a fifth character state. Nodes with <65% bootstrap support (1000 replicates) were collapsed.

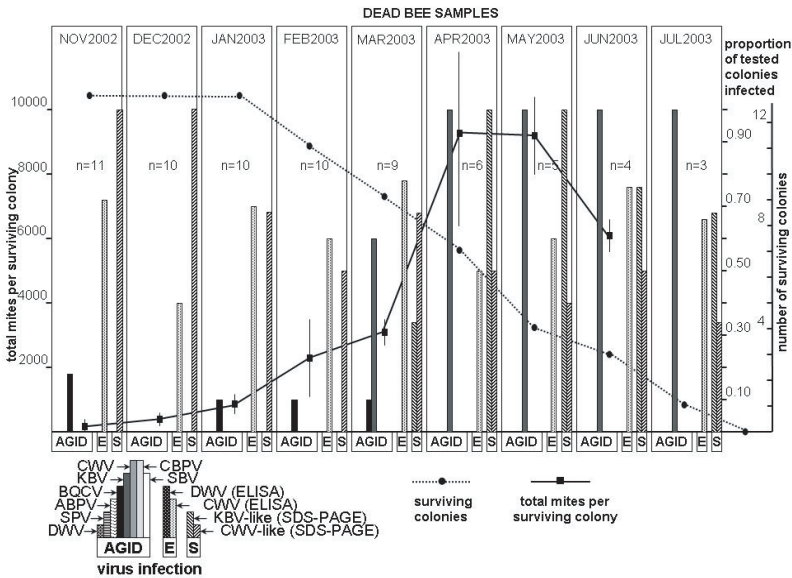
3. RESULTS

3.1. Colony survival and mite incidence

The numbers of colonies surviving during the 2001–2002 and 2002–2003 surveys are summarized in Figures 1a and 1b. For both seasons there was a severe colony failure rate from February and March onwards, coinciding with an increase in the mite population. This increase was estimated only for the 2002–2003 season, where the mean mite population in each colony was significantly greater in April and May than in March ($P < 0.05$; Fig. 1b). The plateau and subsequent decline in mite numbers from April to June 2003 reflects the collapse of the colony and the dearth of bees and brood to sustain the mite population. Five of the colonies that died in February or March 2003 had lost their queen and



(a)



(b)

Figure 1. Incidence of the major honey bee viruses in samples of dead and live bees from dying honey bee colonies during the 2001–2002 (Fig. 1a) and 2002–2003 (Fig. 1b) seasons. The various viruses were detected by agarose gel immunodiffusion (AGID; “S” fraction), ELISA (E; “P” fraction) and SDS-PAGE capsid protein profiling (S), each represented by a differently shaded histogram bar. The samples were tested for the presence of BQCV, SBV, SPV, CBPV, KBV, ABPV, CWV and DWV by AGID, CWV and DWV by ELISA, and for viral capsid protein bands by SDS-PAGE (only KBV-like and CWV-like viruses detected). The number of colonies sampled randomly each month for virus analysis (n) and the rate of colony collapse (dotted line; circles) are also shown. For the 2002–2003 season, the number of mites in each surviving colony was also estimated each month (mean; standard deviation) and plotted (solid line; squares).

were unable to produce a replacement, suggesting the queen stopped producing eggs before it disappeared. Colony decline in all the other colonies invariably began with the appearance of dead brood during early autumn (March and April), with the number of dead larvae increasing progressively until colony death. Such observations indicate a lack of adult bees to clear out dead and diseased brood, and are typical for an epizootic increase in incidence, titre and virulence of (viral) diseases of brood and/or adults. There were no clinical signs of bacterial or fungal brood diseases in the colonies during these experiments.

3.2. Serological and protein profile analysis

Results of the serological and protein profile analyses are shown in Figure 1. There was an increase in the overall virus incidence as the seasons progressed. With the exception of DWV, ABPV and SPV, each of the honey bee viruses tested for were detected at some stage in the colonies by AGID, but there appeared to be no evidence of seasonality or association with colony mortality for any one virus. Interestingly, the dead bee samples of the 2001–2002 season produced greater incidence and variety of viruses than the live bee samples of the same months (Fig. 1a).

The more sensitive ELISA test used for the detection of CWV and DWV detected a higher incidence of CWV than the AGID test using the same samples, while no DWV was detected in any of the samples. CWV was detected in dead bees in most months in almost every colony, irrespective of the size of the mite population. The samples from the 2002–2003 season frequently detected a single 22 kDa protein band on SDS-PAGE, which is similar to the protein profile for CWV (Bailey and Ball, 1991; Fig. 2a). However, the presence of this protein in the “S” extracts is unusual since CWV is usually removed from this extract through its tendency to aggregate and precipitate in the extraction buffer (0.01 M PB pH 6.7). It is also interesting to note that this virus protein profile was rarely seen in the 2001–2002 extracts, although CWV was also abundantly detected by AGID and ELISA in these samples. This 22 kDa protein was often the only protein in the sample revealed by SDS-PAGE, and was not always present in the colonies when they collapsed. To determine whether this band belonged to CWV or some other virus, “S” and “P” extracts,

prepared from live bees collected during the same month as the dead bees displaying the 22 kDa protein, were tested against antisera for BQCV, SBV, SPV, CBPV, ABPV, KBV-Canada, CWV, DWV, bee virus X, bee virus Y, Egypt bee virus and chronic bee paralysis associate virus. Only the CWV antiserum gave positive results. It is likely, therefore, that this 22 kDa protein belongs to CWV, although there appear to be differences between this virus and the CWV-UK characterised by Bailey and Ball (1991) in terms of purification and the apparent size of the capsid protein.

There were also some inconsistencies between the serological and protein profile data for KBV. AGID only detected this virus occasionally in colonies, in April 2002 and from March 2003 onwards. However, the SDS-PAGE data identified with high frequency a virus with a KBV-like protein profile which did not react consistently in AGID with the principal KBV antiserum (KBV-NewZealand) even though AGID is a more sensitive test than protein profiling. The same results were obtained when re-testing the extracts with four alternative KBV antisera (KBV-NZ1, KBV-NZ2, KBV-NZ4, KBV-NZ6). The samples generally had three major protein bands of approximately 38 kDa, 34 kDa and 24 kDa (Fig. 2) which is similar to that of known strains of KBV (Allen and Ball, 1995; Stoltz et al., 1995) and matches very well with the theoretical molecular weights of 37.9 kDa, 33.3 kDa and 23.8 kDa deduced from the published genomic sequence of KBV (de Miranda et al., 2004; Fig. 3). This protein profile was visible in samples from both the 2001–2002 and 2002–2003 seasons and was apparent in the samples collected in the few months preceding the death of most colonies.

The serologically non-reacting KBV-like virus was also detected in the live bee samples collected in the 2001–2002 season. Injection of the head-extract of these bees into test pupae and adult bees caused rapidly fatal infections, with all bees dying within four days, which is also characteristic of KBV. Analysis of extracts from these test adult bees reproduced the KBV-like protein profile (Fig. 2b) of the original extracts (Fig. 2a). Extracts with this protein profile were re-tested by AGID against a wider range of KBV antisera, and at higher antiserum concentrations. They were found to have either no or weak affinity with the ABPV antiserum and a stronger affinity with most of the alternative KBV antisera. The strongest reactions were against the KBV-Spain and KBV-Canada antisera. These data, combined with the recognizable KBV-like

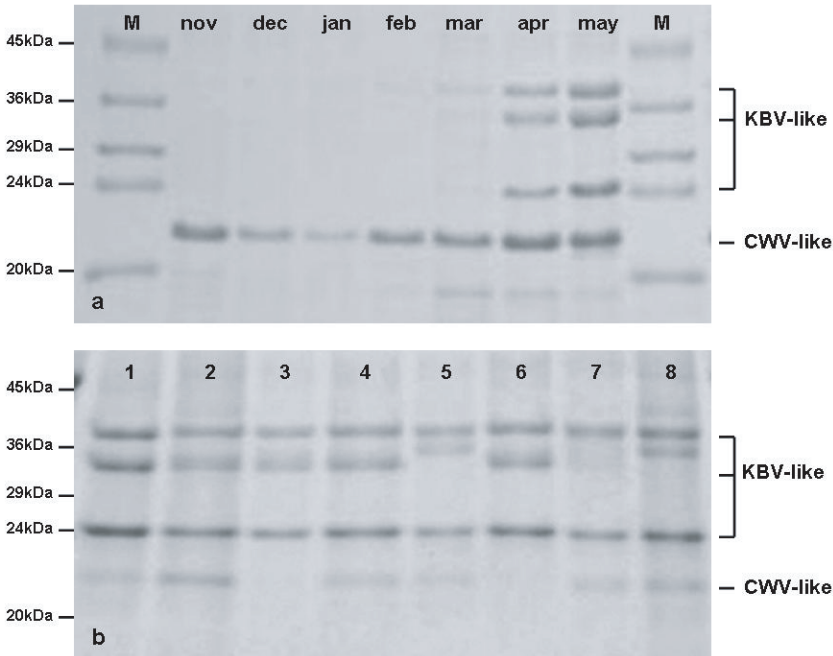


Figure 2. SDS-PAGE gels. (a) Shows the changing composition of the viral capsid protein profile of monthly extracts of bees from a single hive from the 2002–2003 season, with both the KBV-like and CWV-like viral capsid protein bands clearly visible. (b) Reveals the viral capsid protein profile of 8 individual test adult bees that died following injection with extracts made from the heads of live bees from collapsing New Zealand colonies. Note that the CWV-like viral capsid protein band is not always present in these samples. The variation in the size of the KBV-like protein bands between the samples is common for strains of KBV (Allen and Ball, 1995).

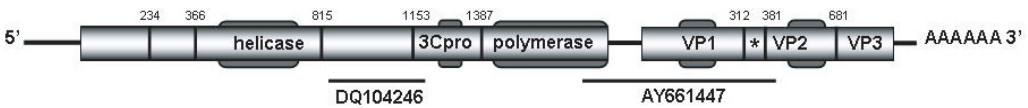


Figure 3. Map of the KBV genome showing the location of the KBV-New Zealand sequences, identified by their GenBank accession numbers (DQ104246 and AY661447) with respect to the predicted (3C)-protease digestion sites, and the major functional domains: the helicase domain (helicase), the 3C-protease domains (3Cpro) the RNA polymerase domains (polymerase) and the virus particle proteins VP1, VP4 (identified by an asterisk *), VP2 and VP3.

protein profile, provisionally identified this abundant virus as a serologically distinct variant of KBV that is most closely related to the KBV strains from Canada and Spain.

3.3. RT-PCR, sequence and phylogenetic analyses

Composite bee and mite samples from a severely mite-infested, moribund colony were analysed by

RT-PCR for the presence of KBV, ABPV and DWV. Only amplifications with primer sets 1 and 3 (designed to detect both ABPV and KBV, or any variant in between), and primer sets 2, 4 and 5 (specific only for KBV) produced a positive signal. No amplification was observed with either of the DWV-specific primer sets (6 and 7), indicating no DWV could be detected in these colonies. The KBV/ABPV RT-PCR products were initially cloned, rather than sequenced directly, to allow for the possibility of multiple and divergent virus

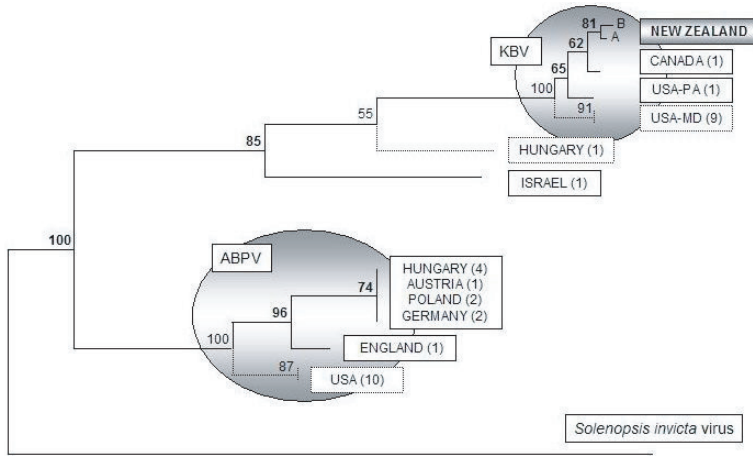


Figure 4. Phylogenetic analysis of a 226 nucleotide section located in the VP4 region. The dotted lines indicate the positions of additional taxa, deduced from similar analyses of overlapping taxa covering the VP2 region. Indicated are the confidence data for each node, shown as a percentage of correct partitions, based on 1000 bootstrap replicates. The following GenBank accessions were used in these analyses: KBV-NZ-A (AY661447); KBV-NZ-B (EF203916); KBV-USA-MD (AF263723; AF263725–AF263732); KBV-CANADA (AY452696); KBV-USA-PA (AY275710); “KBV”-Hungary (AF346301); ISRAEL bee virus (AY738529); ABPV-ENGLAND (AF150629); ABPV-Hungary (AY053372–AY053375; AY053377–AY053385; AY059372); ABPV-Poland (AY053371; AY053376); ABPV-E. Europe (AY053366–AY053370); ABPV-USA-FL (AF264688–AF264692); ABPV-USA-MD (AF263724; AF263733–AF263736) and *Solenopsis invicta* virus (AY634314).

strains present in the sample (Evans, 2001). The presence of these strains could render any direct sequence of RT-PCR products difficult to interpret, especially with the gap differences between ABPV, KBV and similar viruses (de Miranda et al., 2004). A minimum of three clones were sequenced per RT-PCR product, yielding a total of 2828 nucleotides of consensus sequence (GenBank accessions DQ104246 and AY661447) in two regions of the genome of a virus closely related to KBV (Fig. 3). There was minimal sequence difference between the replicate clones, suggesting these particular bees, from 2004, were most likely infected with a single virus strain. However, since the sequences derived from cloned RT-PCR products are unrepresentative of the wider genetic diversity within the isolate, the primary RT-PCR products of several virus extracts from 2003 were also sequenced directly. Such sequences are automatically consensual for the entire population of related virus genomes in the sample. Since the global ABPV/KBV primer set-1 was used for amplification, most strains within the sequence space between ABPV and KBV would have been amplified, providing maximum chance for identifying divergent virus strains that may be present in New Zealand. The direct sequences

of RT-PCR products from the 2003 samples displayed minor heterogeneity, reflecting the natural variation within each isolate. This heterogeneity was resolved by cloning the RT-PCR products, followed by sequencing several clones, which revealed the presence of two closely related (98.5% nucleotide identity), co-infecting sequence variants of the New Zealand KBV strain among the 2003 samples. These two variants are illustrated as ‘A’ (the original KBV-NZ sequence) and ‘B’ (GenBank accession EF203916) in Figure 4. Parsimony analyses of the sequences show the New Zealand strain of KBV to be most closely related to the Canadian KBV isolate (Fig. 4), suggesting this is the same strain as that identified serologically in the previous seasons. Beyond that, the strain is related to the USA KBV isolates, positioning this new strain in a distinct group separated from another group of isolates which includes an Australian KBV strain (de Miranda et al., 2004). Again this is supported by the weaker serological reaction of the virus extracts with the KBV-SouthAustralia antiserum.

There were 111 nucleotide differences between the New Zealand and Pennsylvania KBV sequences, distributed evenly over the two regions sequenced, resulting in 96.1% nucleotide

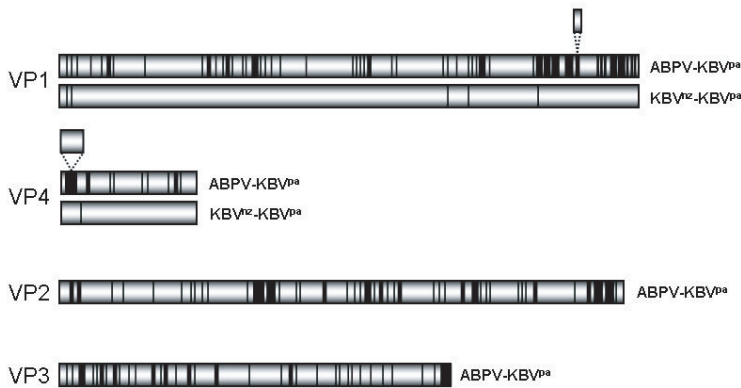


Figure 5. Structural protein variation. Distribution of the amino acid differences, marked with vertical black lines, between the structural proteins VP1, VP4, VP2 and VP3 of ABPV and KBV-PA (Pennsylvania strain), and between those of KBV-NZ and KBV-PA. The structural protein boundaries were identified by the viral protease recognition sites, based on those determined for ABPV (Govan et al., 2000).

identity between them. Despite this uniformity, it is possible that the consensus sequences for the two regions were derived from different, co-infecting KBV strains, both with a similar relationship to the Pennsylvania KBV. Of the nucleotide differences, 104 were located in coding regions, 16.3% of which resulted in amino acid changes, yielding 98.1% amino acid identity between the strains. Of particular interest with respect to the serological variability among strains of KBV and ABPV are the amino acid differences between their structural proteins. Figure 5 shows that the variation between KBV and ABPV structural proteins is heavily concentrated at the C-terminal end of VP1 and the N-terminal end of VP4. Although antisera against ABPV and KBV particles cross-react in Western blot and ELISA (Allen and Ball, 1996), monospecific antisera against the VP4 of these viruses do not (Stoltz et al., 1995). Depending on how these proteins interact to form the virus particle, these variable regions could easily account for the serological differences between ABPV and KBV, and possibly among different KBV strains as well. Since the New Zealand and Pennsylvania KBV isolates are nearly identical in these regions, they almost certainly belong to the same (Canadian) serological subgroup.

4. DISCUSSION

The presence of KBV in dead bees from both the 2001–2002 and 2002–2003 study colonies in the few months preceding their collapse may indicate

a possible link between this virus and the death of mite-infested honey bee colonies in New Zealand. KBV has been implicated in the collapse of infested colonies overseas (Hung et al., 1996) probably as a result of the transmission of the disease by KBV-carrying mites to healthy honey bee pupae during feeding (Chen et al., 2004). The identification of KBV in both mites and dead honey bees from collapsing New Zealand colonies strongly suggests that this virus is being similarly transmitted in New Zealand.

The only other virus found in a significant number of the dead bee extracts was CWV. This virus is a common infection of honey bees (Bailey and Ball, 1991; Ellis and Munn, 2005) and its presence in the 2002–2003 study colonies was irrespective of the presence of mites, a finding which is consistent with other studies (e.g. Nordström et al., 1999). The virus was not always present in the colonies at the time they collapsed, however it was detected in each of the colonies that died following the disappearance of the queens in the 2002–2003 season. Further investigation is required to determine whether this was the cause of the observed queen and colony failures. Molecular characterisation of the virus should reveal whether it is the source of the 22 kDa protein visualised by SDS-PAGE gel in the 2002–2003 dead bee extracts.

BQCV was detected in four of the 2002–2003 colonies during the summer months. This was consistent with the findings of previous studies that have shown that the incidence of this virus is associated with a seasonal increase in the incidence of *Nosema apis* infestation in spring and summer

(Anderson, 1988; Bailey and Ball, 1991), and not related to the presence of mites. BQCV was only present in one of the five colonies that died following the disappearance of the queen bees, suggesting that this infection was not responsible for the observed queen losses.

The difficulty we experienced in detecting the KBV infections in the bee samples using AGID is probably due to serological variation in the capsid proteins of different KBV strains, since the SDS-PAGE analyses revealed more than enough virus for a positive identification by AGID if the viruses were serologically related. Previous studies with this virus have revealed that the protein profiles and serological affinity of different KBV strains vary markedly, both between strains from different countries and between those from within a country or region (Bailey et al., 1979; Allen and Ball, 1995; Stoltz et al., 1995). By using increasingly concentrated KBV antisera, the KBV isolate in the New Zealand bee samples was shown to be serologically most closely related to the Spanish and Canadian KBV strains. Such serological differences are due to conformational and/or genetic differences between the capsid proteins of the viruses. These differences have been mapped for ABPV and KBV (all four capsid proteins) and for VP1 and VP4 of the New Zealand and Pennsylvania strains of KBV (Fig. 5). Phylogenetic analysis of the partial sequences of the New Zealand KBV strain confirmed, at genetic level, the serological relationship of the New Zealand and Canadian KBV isolates, and suggests that both probably descended from a common ancestor with the other North American isolates. These results are similar to those of Siede and Büchler (2004) who, using a different region of the KBV genome for phylogenetic analysis, also located their KBV isolates from New Zealand in the same area of the phylogram, i.e. close to the North American isolates. The phylogram also shows the extent of the variability within the KBV clade, compared with the ABPV clade, especially through the inclusion of the divergent isolates from Hungary and Israel (Fig. 4). These two isolates have an equidistant relationship with each other and the main branch of the KBV clade, as reflected by the low bootstrap number for the node, and may represent a period of radiative evolution in the KBV clade. The inclusion of more isolates in this part of the phylogram may help resolve this node better. This evidence of extensive variability within the ABPV-KBV species complex, particularly at the KBV end of the spectrum, makes it likely that other

divergent strains exist, as is also implied by the serological evidence for KBV. Although the New Zealand KBV strain identified in these samples falls cleanly into a well-established part of the KBV clade, the difficulties encountered reconciling conflicting evidence for KBV-like viruses in this study, plus prior evidence for multiple KBV strains in New Zealand (Anderson, 1985, 1988, 1991), make it well possible that other, more divergent KBV strains will be discovered here.

KBV was an established infection in the study colonies in autumn just prior to their collapse and at a time when mite populations were at their peak. By modelling all currently known virus dynamics, Sumpter and Martin (2004) conclude that virulent, rapidly fatal viruses, such as KBV, can only cause colony death when there are large numbers of mites in the colony. This is because KBV kills bees 3–4 days after the virus is introduced to the haemolymph (Bailey et al., 1979). Virus acquisition and transmission therefore have to proceed at a greater rate than infected bees are lost from the colony. The probability of a mite encountering an infected bee and acquiring KBV, therefore, increases with increasing numbers of mites. Predictions by models such as these are heavily dependent on accurate estimates of comparative viral virulence, i.e. the speed with which different viruses kill bees (brood or adult), since this a critical factor for the successful reproduction of the mite, which transmits the virus. The laboratory estimate for KBV-induced death, which may well be different for varroa-transmitted virus, is 3–4 days after the virus is introduced to the haemolymph (Bailey et al., 1979). In our 2002–2003 study colonies, the mean mite population of >9000 mites per colony in autumn would have been sufficient for the mites to acquire and transmit the infection through these colonies (Sumpter and Martin, 2004). The highly characteristic appearance of the brood cells in these dying colonies, with a lot of brood dying during the early to middle pupal stage, supports the presence of a rapidly fatal pathogen introduced around capping time such as, for example, a mite-transmitted lethal virus.

These results suggest that the loss of New Zealand honey bee colonies may be prevented if acaricides are applied in the late summer or early autumn to prevent the population of *V. destructor* reaching a level which will result in an epidemic outbreak of KBV. Although KBV persisted in the New Zealand honey bee population prior to the arrival of *V. destructor* it was not known to cause

colony death (Anderson, 1985). Thus, although KBV can be spread via mechanisms other than mite to bee transmission, e.g. via excreta (Hung, 2000) or to larvae via KBV-infected food (Anderson and Gibbs, 1989; Shen et al., 2005), reducing the mite population would remove this new, additional vector of the disease and thus may prevent colony collapse. However, this theory is yet to be tested and studies elsewhere (e.g. Ball, 1997; DeGrandi-Hoffman and Curry, 2004) indicate that knowledge of virus epidemiology is essential before effective management or treatment strategies can be developed.

It is particularly interesting that DWV was not detected in any of our study colonies, even with the use of the highly sensitive RT-PCR analysis. DWV now predominates in infested honey bee colonies in many areas of the world (Calderón et al., 2003; Tentcheva et al., 2004b; Chen et al., 2005) and the transmission of this virus by *V. destructor* has been linked to colony collapse in these countries. Occasionally, adult bees with deformed wings were observed in our heavily infested study colonies, but DWV was not detected in these individual bees. It is possible that the deformed wings were caused by the feeding activities of the mites on the bee pupae or, alternatively, DWV may be present in New Zealand honey bees but was not detected in our study. This could be because of the unstable nature of the virus, which would have led to its rapid degradation in the bee samples, or because of the virulence of the KBV infection in these samples. KBV is known to predominate in mixed infections in individual bees because of its more rapid replication rate (Anderson and Gibbs, 1988). It is likely, then, that in bee colonies with large mite populations, KBV will become the most prevalent infection causing colony loss, and may mask the presence of other infections such as DWV. Further investigations of infested New Zealand honey bee colonies should determine whether this is indeed the case, and whether it is likely to cause problems when mite populations are kept under the level required for the development of epizootic KBV infections.

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Incidence et caractérisation moléculaire des virus trouvés dans des colonies d'abeilles domestiques (*Apis mellifera*) mourantes infestées par *Varroa destructor* en Nouvelle Zélande.

Apis mellifera / virus du Cachemire de l'abeille / dépopulation / Nouvelle Zélande / analyse sérologique / SDS-PAGE / RT-PCR

Zusammenfassung – Vorkommen und molekulare Charakterisierung der Viren in absterbenden mit *Varroa infizierten* Bienenvölkern (*Apis mellifera*) in Neuseeland. Beim Saugen an adulten Bienen und an Puppen kann die parasitische Milbe *Varroa destructor* verschiedene Honigbienenviren über Bienenpopulationen hinweg verbreiten. Diese Viren verursachen Infektionen, die zum Tod oder zu Missbildungen bei der infizierten Brut und adulten Bienen führen können, und damit letztendlich zum Absterben der betroffenen Bienenvölker. Vier Bienenviren konnten bereits mit Völkerverlusten in Europa und den USA in Zusammenhang gebracht werden: das Kaschmir-Bienenvirus (KBV), das Akute-Bienenparalysevirus (ABPV), das Langsame-Paralysevirus (SBV) und das Verkrüppelte-Flügelvirus (DWV). Wir benutzten eine Reihe von Methoden, um nachzuweisen, welche dieser Viren in Milben und absterbenden Völkern in Neuseeland vorkommen. Von 30 im November 2001 etablierten Völker waren im Mai 2002 alle tot, und weitere 13 im November 2002 etablierte Völker starben im August 2003. Bereits erste serologische Untersuchungen wiesen vermehrt auf einen Befall mit dem Trübe-Flügelvirus (CWV) hin (Abb. 1a und 1b). Aber dieses war nicht in allen Fällen mit dem Zusammenbruch jener Völker assoziiert. Mittels einer SDS-PAGE-Analyse von Bienenextrakten konnten wir ein Muster viraler Capsidproteine nachweisen, die dem bekannter KBV-Stämme ähnlich war (Abb. 2a). Dieses Profil stimmte auch mit dem von Bienen überein, die als Folge einer Injektion mit Kopfextrakten lebender Bienen gestorben waren (Abb. 2b). AGID (Agar-Gel-Immunodiffusion) Tests mit steigenden Konzentrationen von Antiseren gegen verschiedene KBV-Stämme ergab positive Reaktionen, was darauf hinwies, dass das Virus ein neuseeländischer KBV-Stamm (KBV-NZ) sein könnte. Das Virus

etablierte sich in der Bienenpopulation im späten Sommer und Herbst, als die Milbenpopulation in diesen Völkern hoch war (Abb. 1b). Eine RT-PCR (reverse-Transkriptions-Kettenpolymerase-reaktion) Analyse mit Milben aus zusammenbrechenden Völkern (mithilfe der in Tab. I aufgelisteten Primer) detektierte eindeutig ein KBV-Produkt und wies daraufhin, dass die Milben das Virus verbreiteten. Das Genom dieses KBV-NZ Stamms wurde kloniert und partiell sequenziert (Abb. 3) und eine phylogenetische Analyse zeigte, dass es eng verwandt ist mit KBV-Isolaten aus Kanada und den USA (Abb. 4). Eine Direktsequenzierung der RT-PCR-Produkte der Virusextrakte aus 2002–2003 resultierte in zwei eng verwandten Sequenzvarianten (A und B; Abb. 4) des KBV-NZ Stamms. Unterschiede in der Aminosäuresequenz der Strukturproteine zwischen dem KBV-NZ und dem KBV-Pennsylvanien Stamm (Abb. 5) könnten der Grund sein für das unterschiedliche serologische Verhalten dieser Virusisolate. Die Ergebnisse zeigen, dass KBV-Infektionen eine wichtige Rolle spielen und grosse Schäden in *Varroa destructor* befallenen neuseeländischen Bienenvölkern verursachen. Der Virulenzgrad dieser Infektion deutet an, dass eine hohe Milbenpopulation erforderlich ist, um eine Infektion in dem entsprechenden Volk aufrechtzuerhalten. Eine Kontrolle der Milbenpopulation sollte dementsprechend ein aussichtsreiches Verfahren sein, um den Ausbruch epidemischer KBV-Infektionen zu verhindern. Weitere Untersuchungen werden notwendig sein, um diese Hypothese zu testen. Interessanterweise fanden wir selbst mittels sensitiver RT-PCR-Methoden kein Verkrüppelte-Flügelvirus in Bienen- und Milbenproben aus Neuseeland.

Apis mellifera / Kaschmir-Bienenvirus / *Varroa destructor* / Neuseeland

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