Journal of General Virology (2005), **86**, 3419–3424 DOI 10.1099/vir.0.81401-0

RT-PCR analysis of *Deformed wing virus* in honeybees (*Apis mellifera*) and mites (*Varroa destructor*)

Constanze Yue and Elke Genersch

Institute for Bee Research, Friedrich-Engels-Str. 32, D-16540 Hohen Neuendorf, Germany

Correspondence
Elke Genersch
elke.genersch@rz.hu-berlin.de

Received 3 August 2005
Accepted 26 August 2005

Deformed wing virus (DWV) is a honeybee viral pathogen either persisting as an inapparent infection or resulting in wing deformity. The occurrence of deformity is associated with the transmission of DWV through *Varroa destructor* during pupal stages. Such infections with DWV add to the pathology of *V. destructor* and play a major role in colony collapse in the course of varroosis. Using a recently developed RT-PCR protocol for the detection of DWV, individual bees and mites originating from hives differing in Varroa infestation levels and the occurrence of crippled bees were analysed. It was found that 100 % of both crippled and asymptomatic bees were positive for DWV. However, a significant difference in the spatial distribution of DWV between asymptomatic and crippled bees could be demonstrated: when analysing head, thorax and abdomen of crippled bees, all body parts were always strongly positive for viral sequences. In contrast, for asymptomatic bees viral sequences could be detected in RNA extracted from the thorax and/or abdomen but never in RNA extracted from the head. DWV replication was demonstrated in almost all DWV-positive body parts of infected bees. Analysing individual mites for the presence of DWV revealed that the percentage of DWV-positive mites differed between mite populations. In addition, it was demonstrated that DWV was able to replicate in some but not all mites. Interestingly, virus replication in mites was correlated with wing deformity. DWV was also detected in the larval food, implicating that in addition to transmission by *V. destructor* DWV is also transmitted by feeding.

INTRODUCTION

Deformed wing virus (DWV) is a plus-strand RNA virus, pathogenic to honeybees. Clinically diseased animals are characterized by morphological abnormalities including malformed appendages, shortened abdomens and mis-colouring. The causal relationship between these symptoms and DWV infection has been demonstrated by injection bioassays using young pupal bees (Bailey & Ball, 1991). DWV, like many other honeybee viral pathogens, generally persists as a latent infection with no apparent symptoms. Outbreaks of clinical DWV infections have been reported to be associated with Varroa infestations (Ball & Allen, 1988; Martin *et al.*, 1998; Martin, 2001). The role of the ectoparasitic mite, *Varroa destructor*, in the transmission of DWV has been confirmed experimentally under field conditions (Bowen-Walker *et al.*, 1999). Furthermore, it was concluded that it is the level of DWV present in the bees that determines whether they are deformed at emergence, rather than just the presence or absence of virus (Bowen-Walker *et al.*, 1999). Since crippled bees are not capable of surviving, infections with DWV add to the pathology of *V. destructor* and play a major role in honeybee colony-collapse in the course of varroosis, as evident from field observations and supported by a modelling approach (Martin, 2001).

In the past, studies on the incidence and prevalence of DWV infections were hampered by the fact that only traditional techniques that were low in sensitivity and specificity were available. Recently, publication of the complete nucleotide sequences of the genome of DWV (GenBank accession nos. NC_004830 and AY292384), led to the development of several RT-PCR protocols for the detection of DWV (Tentcheva *et al.*, 2004a, b; Genersch, 2005; Chen *et al.*, 2005). Using RT-PCR, DWV could be detected in all life stages of the honeybee, i.e. from eggs to adult bees, and irrespective of wing deformities, as well as in the parasitic *V. destructor* mites (Chen *et al.*, 2005). Tentcheva *et al.* (2004b) demonstrated a higher titre of DWV in bees with deformed wings compared with asymptomatic ones (no deformed wings). A more detailed analysis of DWV infection in deformed and asymptomatic bees, drones, pupae and larvae by quantitative RT-PCR revealed differences in viral concentrations, with pupae having the highest concentration followed by deformed bees, larvae, normal bees and drones (Chen *et al.*, 2005). Both studies confirm earlier reports on

the correlation between the level of infection and the occurrence of clinical symptoms (Bowen-Walker *et al.*, 1999). So far, only quantitative differences between healthy-looking and crippled bees have been analysed and demonstrated. Here, we report qualitative differences for the first time. We studied asymptomatic and deformed bees, but instead of taking the whole bee we analysed the head, thorax and abdomen separately. We also differentiated between the detection of plus- and minus-strand viral RNA to demonstrate replicating and non-replicating virus. This analysis was not only performed with bees but also with mites collected from differentially infested colonies and collected from crippled and asymptomatic bees.

METHODS

Collection of honeybees, mites and larval food. Honeybees and mites were collected from hives (19, 332, 357, WV) of the Institute's apiary during the years 2003 and 2004 (see Table 2). Honeybees were collected either at the time of emergence (freshly-hatched bees) or from the honeycombs and the hive entrance (older bees). At least 40 bees (four groups of 10: freshly-hatched crippled and healthy, older bees crippled and healthy) per hive were analysed. Since *V. destructor* is endemic in Germany all hives were infested by *V. destructor*, although infestation levels and the occurrence of clinical symptoms of DWV infection (emerging crippled bees) differed (see Table 2). Infestation grade was determined by the end of July by counting the number of dead mites falling down onto the base of the hive during treatment with formic acid (a flask filled with 100 ml 80 % formic acid and equipped with a paper wick to facilitate evaporation of the formic acid; put into the hive for 14 days). Twenty-four bees from three hives (Swe 1-3) from a region of northern Sweden, where Varroa has not yet arrived (see Table 2), were a generous gift from Professor Ingemar Fries (SLU, Uppsala, Sweden). Mites were collected alive from freshly-hatched or older bees and combs, and put singly into reaction tubes. At least 40 individual mites per hive were analysed (two groups of 10: mites from freshly-hatched crippled and healthy bees; one group with 20 mites collected from older bees and the combs). Larval food was collected from uncapped cells of larvae, no older than 2 days. Bees, mites and larval food were frozen at −70 °C immediately after sampling, and stored at −70 °C until total RNA was extracted.

RNA extraction. Frozen bees were dissected into head, thorax and abdomen using a fresh scalpel for every cut to avoid cross-contamination of viral RNA. Total RNA of each body part was extracted using standard methods following the manufacturer's protocol (RNeasy kit; Qiagen). RNA extraction from larval food (30 µl each) was performed by standard methods following the manufacturer's protocol (Viral RNA kit; Qiagen). Total RNA from individual mites was extracted using the Purescript RNA extraction kit (GentraSystems) following the manufacturer's protocol with minor modifications. Briefly, single mites still frozen at −70 °C were crushed with a pestle pre-frozen at −70 °C and homogenized in 100 µl lysis buffer. DNA precipitation buffer was added (33 µl) and probes were incubated on ice for 5 min, prior to centrifugation at 16 000 ***g*** for 3 min to pellet the DNA. RNA was precipitated from the supernatant by adding 100 µl 100 % 2-propanol and a subsequent centrifugation step at 16 000 ***g*** for 3 min. The RNA pellet was air-dried for 15 min and washed with 70 % ethanol. The air-dried RNA pellet was resuspended in 18 µl hydration buffer and stored at −70 °C.

One-step RT-PCR for the detection of DWV RNA. One-step RT-PCR was performed according to standard protocols (One-step RT-PCR kit; Qiagen) and as previously described (Genersch, 2005). The following temperature scheme was used: 30 min at 50 °C, 15 min at 95 °C followed by 35 cycles with 30 s at 94 °C, 1 min at 54·3 °C, 30 s at 72 °C, each, including a final elongation step for 10 min at 72 °C. Using the primer pair F15/B23 (Genersch, 2005; see Table 1) a product of 451 bp (position 9247–9697; positions refer to GenBank accession no. NC_004830) was amplified. PCR products (5 µl per reaction) were analysed on a 1·0 % agarose gel. The agarose gel was stained with ethidium bromide and visualized by UV light. Specificity of the amplicons was further verified by sequencing (Medigenomix) random samples.

Tagged RT-PCR for the highly specific detection of plus- and minus-strand DWV RNA. The standard protocol of the one-step RT-PCR (One-step RT-PCR kit; Qiagen) allows for the reverse transcriptase (RT) reaction to be conducted between 50 and 60 °C, due to a special buffer and the mixture of thermostable enzymes Omniscript and Sensiscript included in the kit. This elevated reaction temperature should already ascertain higher strand specificity than achieved with standard protocols (Laskus *et al.*, 1998). In order to further improve strand-specific detection of DWV-RNA, a modified one-step RT-PCR protocol was developed, combining the benefits of an RT reaction at higher temperature and tagged primers (Craggs *et al.*, 2001). RT reactions were performed at 55 °C in the presence of tagged primers tag-B23 or tag-F15 (Table 1). At the end of the RT reaction the PCR machine was paused, the primer pairs Tag/F15 (for tag-B23) or Tag/B23 (for tag-F15) were added (Table 1), and the programme was allowed to continue the PCR reaction (15 min at 95 °C followed by 35 cycles with 30 s at 94 °C, 1 min at 54·5 °C, 30 s at 72 °C, each, including a final elongation step for 10 min at 72 °C). PCR products (7·5 µl per reaction) were analysed on a 1·0 % agarose gel. The agarose gel was stained with ethidium bromide and visualized by UV light.

Table 1. Nucleotide sequence of primers used in RT-PCR

Primer	Position of 5′-end of DWV primer*	Nucleotide sequence (5′–3′)
F15	9247	TCCATCAGGTTCTCCAATAACGGA
B23	9697	CCACCCAAATGCTAACTCTAAGCG
tag-F15	9247	agcctgcgcaccgtggTCCATCAGGTTCTCCAATAACGGA
tag-B23	9697	agcctgcgcaccgtggCCACCCAAATGCTAACTCTAAGCG
Tag		agcctgcgcaccgtgg

*Numbering according to Lanzi & Rossi (unpublished); GenBank accession no. AJ489744.

Table 2. Percentage of DWV-positive mites and bees from different hives differing in the level of Varroa infestation

Hive #	No. dead mites after treatment with formic acid	Estimated DWV-positive mites (%)	Crippled bees	Estimated DWV-positive bees (%)
19	9	100	Not observed	100
332	2402	45	Not observed	100
357	2048	100	Yes	100
WV	50	100	Not observed	100
Swe 1-3	No mites	No mites	Not observed	40

The chosen tag showed no similarity with any known bee pathogen or invertebrate sequence as verified by processing the tag sequence through BLAST algorithm (Altschul *et al.*, 1990) available on the NCBI website.

RESULTS

Varroa infestation and occurrence of wing deformities

V. destructor is endemic in Germany. Accordingly, all German bees analysed in this study originated from Varroa-infested hives. Infestation grade of these hives differed, as calculated from the number of dead mites after treatment with formic acid. Bees with deformed wings could only be detected in one hive. Also included in this study were bees from a region of northern Sweden from where *V. destructor* infestation has not been reported so far. Therefore, these hives were Varroa-free (Table 2). While 100 % of the German bees analysed were positive for DWV, in 60 % of the Swedish bees no sequences of DWV could be detected (Table 2).

Virus detection in honeybees

Since we could not confirm quantitative differences in virus titres correlating with the occurrence of wing deformities, we looked for qualitative differences between crippled and apparently healthy bees. Analysing the spatial distribution of DWV by separately analysing head, thorax and abdomen of crippled and asymptomatic bees for the presence of viral RNA, revealed that all body parts of bees showing deformed wings were always strongly positive for DWV (Fig. 1a, lanes 1–3). In contrast, DWV could never be detected in total RNA extracted from heads of asymptomatic bees (Fig. 1a, lanes 4 and 7). The only bees that were absolutely devoid of DWV originated from a region of northern Sweden (Fig. 1a, lanes 10–12). Detection of viral RNA in the thorax and abdomen of asymptomatic bees differed considerably, although all bees looked the same (Fig. 1b).

Detection of virus replication in honeybees

Since DWV is a plus-strand RNA virus, the minus-strand RNA is only present during replication. Therefore, detection of the minus-strand RNA via a minus-strand-specific RT-PCR is indicative of replication. Analysis of crippled bees for the presence of minus-strand viral RNA demonstrated replicating virus in the head, thorax and abdomen (Fig. 2a, lanes 1–6), while only faint bands for minus-strand viral RNA could be detected in asymptomatic bees, showing signals for plus-strand viral RNA only in total RNA from abdomen (Fig. 2a, lanes 7–12). Expectedly, no replicating virus could be detected in larval food, although plus-strand viral RNA was present (Fig. 2a, lane 13). Analysing several asymptomatic bees that were strongly positive for plus-strand viral RNA in both thorax and abdomen revealed great variability in virus replication but no correlation between the amount of plus-strand RNA and the level of replication (Fig. 2b).

Detection of DWV in mites

Since Varroa-infestation level and the occurrence of clinical symptoms of DWV infection (emerging bees with deformed wings) did not always correlate (Table 2), we hypothesized that the proportion of DWV-transmitting mites differed between the hives studied. Analysing mites individually for the presence of viral RNA revealed that indeed the percentage of DWV-positive mites was different from hive to hive and

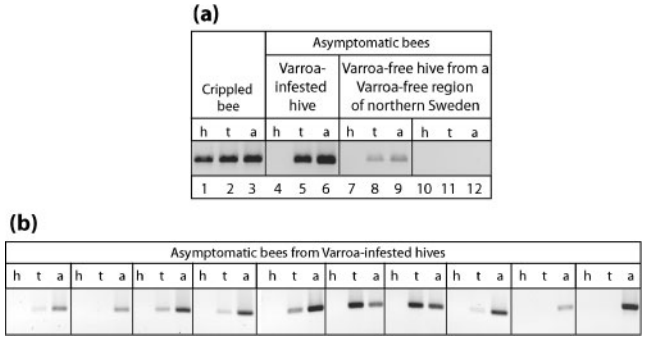

Fig. 1. Detection of DWV in crippled and asymptomatic bees. (a) Total RNA extracted from the head, thorax and abdomen (h, t and a, respectively) of bees with deformed wings and asymptomatic bees was analysed by one-step RT-PCR for the presence of DWV. (b) Total RNA extracted from the head, thorax and abdomen of 10 randomly sampled asymptomatic bees was analysed by one-step RT-PCR for the presence of DWV.

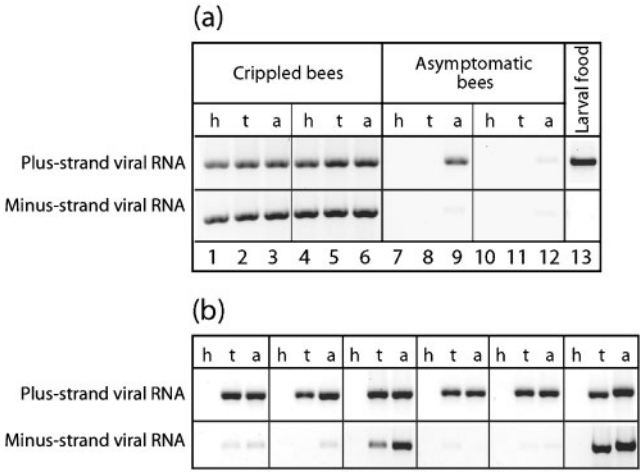

Fig. 2. Specific detection of plus- and minus-strand viral RNA using tagged primers for RT-PCR. (a) Using a modified one-step RT-PCR protocol, total RNA extracted from the head, thorax and abdomen (h, t, a, respectively) of two crippled and two asymptomatic bees was analysed for the presence of plus- and minus-strand DWV RNA. Larval food was also analysed for the presence of plus- and minus-strand DWV RNA. (b) Using a modified one-step RT-PCR protocol, total RNA extracted from the head, thorax and abdomen of six asymptomatic bees strongly positive for DWV was analysed for the presence of plus- and minus-strand DWV RNA.

did not correlate with infestation level. The level of DWV-positive mites ranged from 45 to 100 % between mite populations infesting different hives (Table 2 and Fig. 3).

Detection of DWV replication in mites

Even in a hive with 100 % DWV-positive *V. destructor* mites, pupae parasitized by such mites do not inevitably develop into adult bees showing deformed wings. Therefore, we speculated that mite subpopulations differing in virulence might exist. We analysed individual mites collected from freshly-hatched crippled and asymptomatic bees. All mites collected were strongly positive for DWV (Fig. 4, upper panel). Analysis of virus replication revealed that all mites collected from crippled bees contained considerable amounts of actively replicating virus. In contrast, virus replication in mites collected from asymptomatic bees was not detectable (Fig. 4, lower panel).

Fig. 3. Variation in the proportion of DWV-positive mites between different mite populations. Using a one-step RT-PCR protocol, mite populations originating from hives with similar Varroa-infestation levels (Table 2) were screened for the presence of DWV in individual mites. (a) Mites from hive 357 and (b) mites from hive 332.

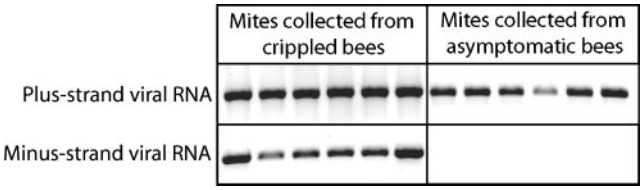

Fig. 4. Specific detection of plus- and minus-strand viral RNA in Varroa mites using tagged RT-PCR. Using a modified one-step RT-PCR protocol, *V. destructor* mites collected from freshly-hatched crippled and asymptomatic bees were individually analysed for the presence of plus- and minus-strand DWV RNA. Representative results are shown.

DISCUSSION

DWV is considered the most serious secondary pathogen associated with varroosis. DWV adds to the pathology of *V. destructor* and is thought to play a role in colony collapse in the course of Varroa infestation (Martin, 2001). The transmission route for DWV through *V. destructor* as a vector is well documented (Bailey & Ball, 1991; Bowen-Walker *et al.*, 1999), although the mechanism leading to wing deformity is still elusive. But since DWV is also present in the absence of *V. destructor*, other transmission routes must exist. Recently, RT-PCR detection of DWV in honeybee eggs has been reported (Chen *et al.*, 2005), suggesting also vertical transmission. Here, we demonstrated that larval food contains DWV, indicating feeding as a third route of transmission. Both routes explain DWV-positive bees in the absence of Varroa, as detected by analysing the Swedish bees originating from a Varroa-free region as well as the phenomenon that we detected 100 % DWV-positive bees even in hives with only 45 % DWV-positive mites. Curiously, Tentcheva *et al.* (2004b) did not detect DWV-positive bees in Varroa-free hives in France, although DWV was shown to be prevalent in Varroa-infested French honeybee colonies.

To date, only quantitative differences in virus titres have been discussed to explain the fact that not all virus-positive bees show clinical symptoms of DWV infection (Bowen-Walker *et al.*, 1999; Nordström, 2003; Tentcheva *et al.*, 2004b). Here, we report qualitative differences between crippled and healthy bees for the first time. Crippled and healthy-looking bees differed in the spatial distribution of DWV. Only crippled bees were positive for DWV not only in the thorax and

abdomen, but also in the head. So far, we have been unable to detect this virus in the heads of asymptomatic bees. This is in contrast to a recent study describing the isolation of a virus 99 % identical to DWV from the brains of aggressive worker bees (Fujiyuki *et al.*, 2004). However, although we did not detect DWV sequences in total RNA isolated from the heads of healthy-looking bees, it is possible that we failed to detect these rare bees simply because they are quickly excluded from the hive due to their aberrant behaviour. Further studies determining the target organs of DWV, for example by *in situ* hybridization and addressing the molecular pathogenesis of DWV, are needed to elucidate the pathomechanism of DWV infection.

Replication of DWV in mites has been demonstrated recently (Ongus *et al.*, 2004). Here, we provide correlative evidence that virus replication in mites is related to the development of wing deformity. It is tempting to speculate that the same viral proteins/protein structures responsible for entering larval cells whose ultimate fate are wings and head organs of the adult bee (e.g. imaginal disks) might allow the virus to enter mite tissue for replication. Following this hypothesis, viruses lacking a certain protein structure differ in virus tropism, resulting in poor or no replication capacity in mites and in inapparent infections in bees (no wing deformity or aggressive behaviour). Further studies analysing the molecular differences between these two viral subpopulations, presumably differing in virulence, are necessary to reveal the responsible factors.

The data on virus replication in healthy-looking honeybees demonstrated that some of these bees are not just a virus reservoir in the hive, but also add to the spreading of the virus by increasing the amount of virus which is offered to mites feeding on adult bees. Since not all mites allow replication of the virus or not all viruses are able to replicate in mites, this is an important factor for the survival of the pathogen.

The sometimes missing correlation between the grade of Varroa infestation, occurrence of clinical symptoms of DWV infection and colony collapse has shed some doubt on the causal relationship between *V. destructor*, DWV and deformed wings (Hung *et al.*, 1996). Here, we present possible explanations for some of the observed inconsistencies. It is conceivable that mite populations differing in their proportion of DWV-positive individuals have a different impact on bee health and colony survival. Under these considerations, the proportion of DWV-transmitting mites is as crucial a figure as the absolute number of mites infesting a colony. In addition, if viral subpopulations differing in virulence exist, as suggested by our data on the correlation between virus replication in mites and wing deformity, then this is another factor influencing the outcome of Varroa infestation that cannot be deduced by just estimating the number of mites infesting a hive.

In conclusion, our data presented show for the first time that (i) differences in the spatial distribution of DWV correlate with wing deformity, (ii) bees with deformed wings and healthy-looking bees differ in the level of virus replication, and (iii) virus replication in mites correlates with viral virulence. Furthermore, our data suggest that an important factor influencing the outcome of Varroa infestation is the proportion of DWV-transmitting mites in the mite population infesting a hive. In addition, the suggested subpopulations differing in virulence will also have an impact on the parasitic mite syndrome.

ACKNOWLEDGEMENTS

This work was supported by the EU (according to regulation 1221/97) and by grants from the Ministries of Agriculture of Brandenburg, Sachsen and Thüringen, Germany, and the Senate of Berlin, Germany.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Bailey, L. & Ball, B. V. (1991). *Honey Bee Pathology*, 2nd edn. London: Academic Press.

Ball, B. V. & Allen, M. F. (1988). The prevalence of pathogens in honey bee (*Apis mellifera*) colonies infested with the parasitic mite *Varroa jacobsoni*. *Ann Appl Biol* **113**, 237–244.

Bowen-Walker, P. L., Martin, S. J. & Gunn, A. (1999). The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. *J Invertebr Pathol* **73**, 101–106.

Chen, Y. P., Higgins, J. A. & Feldlaufer, M. F. (2005). Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). *Appl Environ Microbiol* **71**, 436–441.

Craggs, J. K., Ball, J. K., Thomson, B. J., Irving, W. L. & Grabowska, A. M. (2001). Development of a strand-specific RT-PCR based assay to detect the replicative form of hepatitis C virus RNA. *J Virol Methods* **94**, 111–120.

Fujiyuki, T., Takeuchi, H., Ono, M., Ohka, S., Sasaki, T., Nomoto, A. & Kubo, T. (2004). Insect picorna-like virus identified in the brains of aggressive worker honeybees. *J Virol* **78**, 1093–1100.

Genersch, E. (2005). Development of a rapid and sensitive RT-PCR method for the detection of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*). *Vet J* **169**, 121–123.

Hung, A. C. F., Shimanuki, H. & Knox, D. A. (1996). The role of viruses in bee parasitic mite syndrome. *Am Bee J* **136**, 731–732.

Laskus, T., Radkowski, M., Wang, L.-F., Vargas, H. & Rakela, J. (1998). Detection of hepatitis G virus replication sites by using highly strand-specific Tth-based reverse transcriptase PCR. *J Virol* **72**, 3072–3075.

Martin, S. J. (2001). The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach. *J Appl Ecol* **38**, 1082–1093.

Martin, S., Hogarth, A., van Brenda, J. & Perrett, J. (1998). A scientific note on *Varroa jacobsoni* Oudemans and the collapse of *Apis mellifera* L. colonies in the United Kingdom. *Apidologie* **29**, 369–370.

Nordström, S. (2003). Distribution of deformed wing virus within honey bee (*Apis mellifera*) brood cells infested with the ectoparasitic mite *Varroa destructor*. *Exp Appl Acarol* **29**, 293–302.

Ongus, J. R., Peters, D., Bonmatin, J.-M., Bengsch, E., Vlak, J. M. & van Oers, M. M. (2004). Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. *J Gen Virol* **85**, 3747–3755.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M. E. & Bergoin, M. (2004a). Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. *Appl Environ Microbiol* **70**, 7185–7191.

Tentcheva, D., Gauthier, L., Jouve, S., Canabady-Rochelle, L., Dainat, B., Cousserans, F., Colin, M. E., Ball, B. V. & Bergoin, M. (2004b). Polymerase chain reaction detection of deformed wing virus (DWV) in *Apis mellifera* and *Varroa destructor*. *Apidologie* **35**, 431–439.