Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite

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Viral diseases of honeybees are a major problem in apiculture, causing serious economic losses worldwide, especially in combination with varroa mites. To increase understanding of the relationship among viruses, mites and colony decline, the tripartite relationships among bees, viruses [Kashmir bee virus (KBV) and sacbrood virus (SBV)] and varroa mites have been investigated systematically. To develop an antibody-based test for KBV, two structural recombinant proteins were purified for polyclonal-antibody production. By using ELISA and RT-PCR, the presence of KBV and SBV was studied comparatively in different developmental stages and castes of bees. The results demonstrated that KBV may persist as a viral genome with extremely low levels of viral-capsid proteins and that KBV and SBV can co-infect honeybees. This study indicated the presence of KBV and SBV RNAs in both queens and eggs by RT-PCR, suggesting a route of transovarial transmission. Horizontal transmission is also very likely among adult bees and from adult workers to larvae through contaminated food resources, because both viruses have been detected in all developmental stages and food sources (brood food, honey, pollen and royal jelly). Furthermore, it was demonstrated that mites were another possible route of horizontal transmission, as both viruses were detected in mites and their saliva. This study, for the first time, detected co-occurrence of viruses in varroa, further underlining the importance of the mites in vectoring different bee viruses. Therefore, these results indicated that multiple infection routes exist for honeybee viral diseases.

INTRODUCTION

The honeybee is a eusocial insect; the bee colony is headed by a single queen and is composed of approximately 50 000 individuals (Morse & Hooper, 1985). Many diseases can impact negatively on both feral and domesticated honeybee populations, causing serious economic losses. So far, 18 viruses have been identified from honeybees (Allen & Ball, 1996). Although much research has been done on the characterization of bee viruses, little is known about the transmission routes of bee viruses and the relationship with varroa mites. Disease transmission in the honeybee society may serve as a model system for disease transmission in the human society. Some bee viruses belong to the family Dicistroviridae, which includes Cricket paralysis virus (CrPV). Members of the family Dicistroviridae have some similarities to the picornaviruses. The CrPV proteins are translated from the RNA genome as two separate polyproteins; the translational regulation of the second polyprotein is unique and depends upon an internal ribosome entry site (IRES) at an internal position in the RNA (Jan et al., 2003; Nakashima et al., 1999; Sasaki & Nakashima, 1999, 2000). The CrPV intergenic-region IRES initiates translation by recruiting 80S ribosomes in the absence of initiator Met-tRNA (Jan et al., 2003).

We have focused upon two viruses, Kashmir bee virus (KBV) and sacbrood virus (SBV). The complete nucleotide sequences of KBV and SBV are available (de Miranda et al., 2004; Ghosh et al., 1999). KBV was first identified in adults of the eastern hive bee (Apis cerana) in the northern and western regions of India (Bailey & Woods, 1977). Bees infected with KBV have no described symptoms, even though Bailey & Ball (1991) and Allen & Ball (1995) suggested that KBV is the most virulent of all known honeybee viruses. SBV was first described in 1913, but was not characterized until 1964 (Bailey et al., 1964). SBV is possibly the most common viral disease of bees, being known on every continent (Dall, 1985; Nixon, 1982). SBV infects the brood of the honeybee, resulting in larval death. Larvae with sacbrood fail to pupate and ecdisial fluid, rich in SBV, accumulates beneath their unshed cuticle, forming the sac. SBV may also infect adult bees without any obvious signs of disease. Compared with KBV, SBV causes symptoms that
can confidently be attributed to viral infection (Allen & Ball, 1996).

Varroa was first described as a natural ectoparasitic mite of the eastern honeybee (A. cerana) and then switched host to the western honeybee (Apis mellifera) (Morse & Flottum, 1997). Varroa has now become a serious pest of western honeybees worldwide and is associated with the collapse or death of honeybee colonies. Varroa females initiate reproduction by entering the brood cells of last-stage worker or drone larvae before the cell is sealed. The adult female mite and progeny feed on the haemolymph of pupae and emerge from the cell with the young bee. Varroa mites are also often found on the thorax and abdomen of adult bees and may hitch a ride on bees from a colony to infect other colonies. It is not clear how mites kill bee colonies, but the general assumption is that varroa mites may be vectors or activators of several bee viruses. Some researchers suggest that varroa-mite infestation may be correlated with viral infections (Ball & Allen, 1988; Hung & Shimanuki, 1999; Hung et al., 2000). However, there is little direct evidence to link varroa mites with the occurrence of SBV in adult bees (Ball & Allen, 1988). Multiple opportunities exist for transmission by mites, given the association of the mites with honeybees.

Several methods have been used to detect bee viruses, including immunodiffusion, ELISA, Western blot and RT-PCR (Anderson & Gibbs, 1988; Grabensteiner et al., 2001; Hung & Shimanuki, 1999; Stoltz et al., 1995; Turcu et al., 1994). Diagnosis of bee viruses based on observed symptoms is not always dependable, as bees infected with many viruses may have no symptoms and the viruses are capable of remaining dormant for extended periods of time without any apparent harm to the host. Dall (1985) reported that both KBV and SBV persisted as inapparent infections in seemingly healthy worker-bee pupae and the viruses replicated to detectable concentrations when the pupae were injected with rabbit sera or insect Ringer’s solution. The persistence of inapparent viral infections in honeybees has some similarities to infections by picornaviruses and other viruses within the picornavirus ‘superfamily’. For example, Foot-and-mouth disease virus is a member of the family Picornaviridae and may cause a prolonged, asymptomatic and persistent infection in ruminants (Alexandersen et al., 2002). Girard et al. (2002) reported that Poliovirus persisted in the central nervous system of infected parasylmice for over a year after the acute phase of paralytic poliomyelitis.

Viral transmission between bees is poorly understood. KBV is detected in the faeces of worker and queen honeybees (A. mellifera) (Hung, 2000). The study by Hung (2000) indicated that the queen can be infected, but did not demonstrate direct evidence for transmission via faeces. The queen, under normal conditions, is responsible for laying all of the eggs in the colony (several hundred to thousands per day). It is possible that the queen could transmit viruses to eggs by varroa mite infestation in honeybee eggs. Another route of transmission may be through the food consumed by the larvae and adult bees. The vast majority of individuals in a bee colony are worker bees, which are responsible for feeding the colony. For the first 2 or 3 weeks of adult life, workers stay inside the hive and feed the brood and queen. After this period, the workers forage and bring back nectar, pollen and water. All of these food products are stored and used to feed the larvae, queen and other adults. All of the food, including honey, pollen and royal jelly, is in part composed of secretions from the hypopharyngeal and mandibular glands of the young adult workers. The queen larvae are always mass-fed a diet of royal jelly. Worker larvae are also mass-fed royal jelly for the first 3 days. After the fourth day, the worker larvae are fed, progressively or as needed, with a diet called worker jelly or brood food. This diet consists of royal jelly, honey and probably some pollen. Bailey (1969) showed that more SBV accumulated in the heads and especially in the hypopharyngeal glands of infected worker bees. Therefore, SBV may potentially be transmitted via glandular secretions of the worker bees in the form of food products, but this has not been demonstrated previously.

In this paper, we have developed specific and sensitive methods for diagnosis of KBV infection and studied the presence of KBV in single bees comparatively by ELISA and RT-PCR. To better define the potential transmission routes for bee viruses, we have compared the presence of KBV and SBV in different developmental stages and castes of bees. We have determined whether KBV and SBV could be transmitted from queen bees to eggs via transovarial transmission by testing for viral RNA and capsid proteins in queen bees and viral RNA in egg samples. We were also interested in determining whether food sources (brood food, honey, pollen and royal jelly) were contaminated by these viruses and could be potential sources for horizontal transmission of viruses. In addition, this research provides direct evidence for the potential role of varroa mites in vectoring bee viruses (KBV and SBV). Overall, this research increases our understanding of the complex relationships among honey bees, varroa mites and viral infections.

METHODS

Sample collections. In total, 94 healthy-appearing bees of different developmental stages (larvae, pupae and adults) and castes (queen, worker and drone bees) were collected from 20 colonies in four different apiaries (Penn State, Airport, Hilltop and Rockspring apiaries). The Penn State, Hilltop and Airport apiaries are 4 miles (6.4 km) away from each other and about 30 miles (48 km) away from the Rockspring apiary. Among them, colonies 214 and 216 from the Airport apiary had experienced increased mite levels and SBV infections, but exhibited little or no symptoms of viral infection. Female adult varroa mites were also collected from colony 14.

To develop and validate the methods for KBV detection, 16 5-day-old pupae collected from colony 15 in the Penn State apiary were injected with 4 μl ELISA KBV-positive samples. Fifteen uninjected pupae from...
the same colony served as controls. Both the injected and uninjected pupae were incubated on dry filter paper in small Petri dishes placed in a larger Petri dish lined with filter paper wetted with 12 % glycerol to humidify the air, and were kept in an incubator (35 °C, relative humidity 50 %) for 4 days.

Egg samples were collected carefully from individual cells and washed well with distilled water. For each sample, 20 eggs were pooled and tested. The larval, pupal and adult bees were frozen at −80 °C. To test the presence of viruses in food resources, larval food and royal jelly were collected carefully from cells by removing the worker or queen larvae. Pollen and honey were collected from comb cells.

**Sample preparations.** The larval, pupal and adult bees frozen at −80 °C were cut into half laterally, with one half used for ELISA and the other half for RT-PCR. For ELISA, the half-single-bee samples were homogenized in 400 μl extraction buffer (PBS with 0-2 % diethyldithiocarbamate) and clarified with chloroform. Mites, tested for ELISA, were divided randomly into nine groups of 12 mites each. Extracts from each group of mites in 60 μl extraction buffer were prepared in a similar manner as for the bee samples. Supernatants of the sample extracts were kept on ice and used directly for ELISA or saved at −20 °C for future use. To test whether mite saliva contains KBV, mites were allowed to feed on sterile tissue-culture medium through an artificial membrane (20–25 mites per membrane). After 24 h, the culture medium was collected and assayed for KBV by ELISA.

For RT-PCR, total RNA from the bee samples (pooled eggs, larvae, pupae and adults), mites and food sources (larval food, royal jelly, pollen and honey) was extracted by using TRIzol reagent (Invitrogen). RNA from the egg and mite samples was resuspended in 10 μl DEPC-treated water.

**Expression of KBV proteins in bacteria, and polyclonal antibodies.** Two fragments of KBV, AD and Odt (Fig. 1a), were cloned directionally into pQE-30 (Qiagen). Expression and purification of the recombinant proteins were performed by using Ni-NTA columns under denaturing conditions according to the manufacturer’s instructions (Qiagen). The recombinant proteins were further purified by preparative SDS-PAGE (12 % gel) (Sambrook et al., 1989). The protein bands of interest were excised and electro-eluted. The purified proteins were dialysed in PBS and used to raise polyclonal antisera (Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA, USA). The antisemum against another structural protein, VP₄, was produced against KBV VP₄ (Stoltz et al., 1995) and received as a gift from Dr Don Stoltz, Dalhousie University, Canada.

**ELISA and Western blot.** Ninety-six-well microplates were coated with 50 μl extraction from bees or varroa mites in 150 μl sodium carbonate buffer (coating buffer, pH 9-8) at 4 °C overnight. After rinsing each well three times in PBS-T (PBS with 0-05 % Tween 20), diluted antibodies (1:2000) in PBS-T containing 2 % polyvinylpyrrolidone and 0-2 % BSA were added and the wells were incubated at 37 °C for 3 h. After rinsing each well three times with PBS-T, the wells were incubated with horseradish peroxidase-conjugated protein A (Sigma) (final concentration, 0-08 μg ml⁻¹) at 37 °C for 2 h. Immobilized enzyme was detected colorimetrically by adding the peroxidase substrate 3, 3'-5', 5'-tetramethylbenzidine (0-1 mg ml⁻¹). The end-point A₄₅₀ was measured by using a Spectra Max 250 microplate spectrophotometer (Molecular Devices). Healthy bees negative by RT-PCR for KBV and SBV infections were used as ELISA negative controls. Twice the mean level of reaction of each antibody with healthy bees was subtracted from the absorbance of each sample to correct for the background level of reaction and to set the baseline for a positive reaction. Approximately equal

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**Fig. 1.** Schematic diagram of the genomes of KBV (a) and SBV (b). Open boxes represent open reading frames (ORFs). The nucleotide positions at the beginning and end of each ORF are indicated above the ORFs. Regions of the genome proposed to encode a helicase, protease and RNA-dependent RNA polymerase (RdRp) are indicated. The primers used for RT-PCR (KBV-F and KBV-R, SBV-F and SBV-R) and two expressed KBV proteins are indicated. The predicted sizes and coding regions of KBV capsid proteins are indicated by arrows, along with the predicted N-terminal sequence of each protein and its upstream amino acid residue.
amounts of bee extracts were separated by SDS-PAGE (12 % gel) and transferred to nitrocellulose membranes for Western blot analyses (Harlow & Lane, 1999; Sambrook et al., 1989).

**RT-PCR.** The genomic sequences of SBV (GenBank accession no. AF097294) and KBV (GenBank accession no. AY275710) were aligned by using the Genetics Computer Group (GCG) program, version 10.1. Primers were designed for their specificity to KBV (KBV-F, ATGAGATGATGATGTTCAAG; KBV-R, AATTGCAAGGCCTTGACATC) or SBV (SBV-F, CACCTAACATACACAAAAAG; SBV-R, CATTACTACTCTCACCTTC). Primers (actin-F, ATGAAAGATGTTCGAGACCT; actin-R, TCTGTTTACGAGACTCCCAT) were used to amplify 514 bp of the honeybee actin gene (GenBank accession no. B504901) as an internal control. cDNA synthesis was performed by using moloney murine leukemia virus reverse transcriptase (Promega) and random primers in a volume of 20 μl. RNA from bee samples (2 μg) and 0.5 vol. RNA from bee products (honey, pollen, royal jelly and brood food) and mite samples was used for cDNA synthesis. PCR was carried out by using a program (honey, pollen, royal jelly and brood food) and mite samples was used for cDNA synthesis. PCR was carried out by using a program of initial denaturing for 5 min at 94°C and 35 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 1 min. A 5 μl aliquot of the RT-PCR product was electrophoresed in a 1-5 % agarose gel, stained with ethidium bromide and imaged by using a Kodak digital-image system. Fragment sizes were determined relative to a 100 bp DNA ladder (Promega). A negative control lacking template DNA and a positive cDNA control were performed for each PCR.

**Data analysis.** Student’s t-test (STATVIEW statistical package, version 5.0.1; SAS Institute Inc.) was used to analyse the data.

**RESULTS**

**Development of immunological methods for KBV detection**

To detect KBV capsid proteins, two regions of the KBV genome, AD and Odt, were selected for expression in a bacterial-expression system (Fig. 1a). By using homologous protease-cleavage sites between VP2 and VP6, VP4 and VP3, and VP3 and VP1 in viruses of the family *Dicistroviridae* (Liljas et al., 2002), the cleavage sites of KBV major coat proteins and VP4 were predicted to yield three major polypeptides (VP1, VP2 and VP3) of 23-8, 29-7 and 33-3 kDa, respectively, and a smaller VP4 (7-1 kDa). AD contains the region for capsid proteins VP2, VP3 and the N terminus of VP3, whereas Odt contains VP3 and the N terminus of VP1. Therefore, the two recombinant proteins included all of the major and minor viral-coat proteins (Liljas et al., 2002; Stoltz et al., 1995). Two recombinant proteins of the predicted sizes (41-7 kDa for AD and 27-7 kDa for Odt) were expressed in bacteria and purified by using affinity columns (data not shown). Polyclonal rabbit antibodies were produced against these recombinant proteins.

To test the specificity of the antibodies, we selected nine samples for the detection of KBV by ELISA and Western blot (Fig. 2). Four of the nine samples were positive for capsid proteins by ELISA using anti-VP4. Similar results were obtained by Western blot using the three KBV antibodies. As the recombinant proteins Odt and AD corresponded to more than one putative KBV capsid protein, multiple bands were detected. All of the protein bands detected by anti-AD and anti-Odt antibodies were within the 20–35 kDa size range, which is in agreement with the predicted sizes of the major coat proteins (Liljas et al., 2002) and is also consistent with the sizes of the coat proteins detected in purified KBV separated by SDS-PAGE (Stoltz et al., 1995). However, anti-AD reacted with VP2 and VP3 but, surprisingly, did not react with VP4. Anti-Odt reacted with VP3 and VP1. Anti-VP4 reacted with only VP4. The results demonstrated that the three KBV antibodies were specific for KBV capsid proteins, did not react with bee proteins and did not cross-react with SBV. The low level (<25 %) of amino acid identity between the structural proteins of KBV and SBV, as determined by BLAST analysis, may have precluded the cross-reactivity of the antisera.

**Detection of bee viruses by RT-PCR**

By using specific primers for two honeybee viruses, we detected KBV and SBV in bee samples by using RT-PCR.
Fig. 3. RT-PCR detection of bee viruses. The same samples as in Fig. 2 were tested by RT-PCR using KBV- and SBV-specific primers (lanes 1–9). RT-PCR of bee actin was included as an internal control for the quality of bee RNAs. P, PCR-positive control using cloned KBV and SBV RT-PCR products; N, no template DNA in the reaction as negative control.

(Fig. 3). Bee actin primers were used as an internal standard for RT-PCR and also to demonstrate the quality of the RNA. Primers for KBV, SBV and bee actin were specific and consistently amplified 290, 211 and 514 bp cDNA fragments, respectively. Sequencing of the KBV and SBV cDNA fragments verified their respective identities. In addition to the four bees that were positive for KBV (Fig. 2) by immunological detection methods, two more samples tested positive by RT-PCR (Fig. 3). This study also revealed two bees with KBV and SBV co-infections.

To further compare these methods of KBV detection, we injected 16 healthy-appearing bee pupae with extracts of honeybees that tested positive for KBV infection by ELISA using the anti-VP4 antiserum. In the control group, the three anti-KBV antisera detected 0–40 % KBV positivity whereas, in the injected group, KBV positivity increased to >80 % (81·2–93·8 %), suggesting KBV replication in the injected bees (Table 1). In contrast, RT-PCR detected the presence of KBV in 100 % of bees in both groups. These results demonstrated that the healthy-appearing bees had latent viral infection that could be detected more efficiently by RT-PCR than by ELISA. Furthermore, even though the sensitivity of the three antisera after viral injection did not differ significantly, their sensitivity varied in bees with latent infections. It is also noted that, in this bee colony, ~50 % of the bees had SBV infections and most, if not all, occurred as KBV/SBV co-infections.

Survey of viral prevalence in different bee populations

We applied these detection methods for KBV and SBV to 94 bee samples collected in four different apiaries (Table 1). The proportion of ELISA KBV-positive samples ranged from 3-3 to 50 %, and the proportion of RT-PCR KBV-positive samples ranged from 66-7 to 100-0 %. Except for the Rockspring apiary, all apiaries had high levels of KBV infection (>94 % RT-PCR positivity) and 41-2–88-9 % SBV infection. The Rockspring apiary had the lowest level of KBV infection (66-7 %) and no SBV was detected. This survey further confirmed the presence of viral infections in most apiaries, even though most of the colonies did not show any viral-disease symptoms. It further proved that RT-PCR is a more powerful method for KBV detection in honeybees. This survey also revealed the presence of KBV and SBV co-infections in 38·3 % (36/94) of the tested samples, although the co-infection rates varied between locations of the apiaries.

Transmission routes of honeybee viruses

To test whether bee viruses (KBV and SBV) could be transmitted vertically from the queen to the eggs, we collected 21 queens from the 20 colonies and three egg samples from two of these 20 colonies (colonies 216 and 14). Both KBV and SBV RNA fragments were amplified from queen and egg samples (Table 2), indicating that these viruses may be transmitted from the queen to the eggs via transovarial transmission. KBV RNA was amplified in 71·4 % (15/21) of the queens and SBV RNA was amplified in 61·9 % (13/21)

Table 1. Comparison of KBV and SBV infection rates in injected/un injected pupae and 94 bee samples from four different apiaries from summer and autumn 2002

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample (n)</th>
<th>KBV-positive by ELISA (%)</th>
<th>KBV-positive by RT-PCR (%)</th>
<th>SBV-positive by RT-PCR (%)</th>
<th>Co-infection (KBV and SBV) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-VP4</td>
<td>Anti-Odt</td>
<td>Anti-AD</td>
<td>Anti-VP4</td>
</tr>
<tr>
<td>Injected pupae</td>
<td>16</td>
<td>87·5</td>
<td>81·2</td>
<td>93·8</td>
<td>100</td>
</tr>
<tr>
<td>Uninjected pupae</td>
<td>15</td>
<td>0</td>
<td>6·7</td>
<td>40·0</td>
<td>100</td>
</tr>
<tr>
<td>AP apiary</td>
<td>17</td>
<td>23·5</td>
<td>50·0</td>
<td>NA</td>
<td>94·1</td>
</tr>
<tr>
<td>HT apiary</td>
<td>9</td>
<td>33·3</td>
<td>22·2</td>
<td>11·1</td>
<td>100·0</td>
</tr>
<tr>
<td>PS apiary</td>
<td>38</td>
<td>28·9</td>
<td>37·8</td>
<td>23·7</td>
<td>97·4</td>
</tr>
<tr>
<td>RSP apiary</td>
<td>30</td>
<td>3·3</td>
<td>3·3</td>
<td>NA</td>
<td>66·7</td>
</tr>
</tbody>
</table>

(Ninety-four samples belonging to different stages and castes of bees were from four different apiaries (AP, Airport; HT, Hilltop; PS, Penn State; RSP, Rockspring). Individual bees were divided in half and assayed simultaneously for KBV RNA by RT-PCR and capsid proteins by ELISA using three antisera (anti-Odt, anti-AD and anti-VP4). NA, Not assayed.)
of the queens, indicating that the majority of queens had KBV and SBV co-infections. As several of the queens were known to be 2 years old, viral infections might not have affected their longevity.

Given the role of workers in providing for and feeding the colony, we asked whether the food itself could be a route of viral transmission. The two colonies (14 and 216) tested have a high prevalence of KBV and SBV detected by RT-PCR (100% KBV-positive, 81–90% SBV-positive) (Table 3). This result suggests that worker bees may be partially responsible for the horizontal transmission of both viruses in bee colonies. From these two colonies, we tested several food sources that contain hypopharyngeal- or mandibular-gland secretions for the presence of the two viruses by RT-PCR. In our study, 33–80% of the food sources tested were positive for KBV and 50–100% of these food sources tested positive for SBV (Table 3). In these food sources, co-occurrence of KBV and SBV ranged from 33 to 75%. Bee actin was present in two of five samples of brood food, suggesting that cells from a honeybee were present. Perhaps cell sloughing was occurring in either the worker mandibular glands or in the larva itself. This result suggests that worker bees may be transmitting viruses to larvae or other adult bees via these secretions.

**Varroa mites potentially vector KBV and SBV during parasitization of bees**

Varroa mites feed on the haemolymph of the developing pupae and the adult bees. Female mites enter the brood cells at the end of larval stages and secrete saliva into the host during feeding. This is a potential transmission route for the bee viruses. Mites were collected from the adults or pupae of bees in colony 14. Both KBV and SBV RNAs and KBV viral proteins were detected in mite samples (Fig. 4). Viral RNA could be detected in single mites (Fig. 4a). However, the presence of these viruses was variable in mite samples taken from a single colony, indicating that not all mites had the viruses. KBV was detected in more of the mite samples than SBV. For ELISA, the levels of detected KBV capsid proteins were significantly different between mites and ELISA buffer control (P=0.002, pairwise Student’s t-test) (Fig. 4b).

The direct evidence for the ability of the mites to vector the viruses was the detection of KBV in mite saliva. These mites were taken from colony 14 in the Penn State apiary, which had known KBV and SBV infections. The levels of KBV capsid proteins were significantly different between mite saliva and medium alone (P=0.039, pairwise Student’s t-test) (Fig. 4b), indicating the presence of KBV in the mite secretions.

**DISCUSSION**

Diagnosis of KBV and SBV based on observed symptoms is not dependable, as honeybees infected with KBV and SBV have no reliable symptoms. Developing specific methods for the detection of KBV infection is important. We have compared ELISA and RT-PCR for detection of KBV infection in bees. All three KBV antibodies, which were specific for capsid proteins, did not react with bee proteins and did not cross-react with SBV. The results of our experiments have shown consistently that RT-PCR was much more sensitive than ELISA, and RT-PCR was the method of choice for detecting viral infections. Especially in the survey study, the overall percentage of KBV positivity being positive by RT-PCR.

**Table 2. Vertical transmission of bee viruses (KBV and SBV) from the queen to the eggs**

Anti-VP4 was used in ELISA tests. The number of samples that were positive out of the total number tested is shown in parentheses. NA, Not assayed.

<table>
<thead>
<tr>
<th>Stage</th>
<th>KBV-positive by:</th>
<th>SBV-positive by RT-PCR</th>
<th>Co-infection of KBV and SBV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>RT-PCR</td>
<td></td>
</tr>
<tr>
<td>Queen adults</td>
<td>19·0% (4/21)</td>
<td>71·4% (15/21)</td>
<td>61·9% (13/21)</td>
</tr>
<tr>
<td>Eggs*</td>
<td>NA</td>
<td>66·7% (2/3)</td>
<td>66·7% (2/3)</td>
</tr>
</tbody>
</table>

*Twenty eggs per sample.

**Table 3. Detection of bee viruses (KBV and SBV) in bee food sources and workers (adults and larvae) by RT-PCR**

Bee food sources and workers (adults and larvae) were isolated from two colonies in different apiaries independent from each other. The number of samples that were positive out of the total number tested is shown in parentheses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>KBV-positive</th>
<th>SBV-positive</th>
<th>Concurrency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bee products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Honey</td>
<td>75·0% (3/4)</td>
<td>75·0% (3/4)</td>
<td>75·0% (3/4)</td>
</tr>
<tr>
<td>Pollen</td>
<td>33·3% (1/3)</td>
<td>100·0% (3/3)</td>
<td>33·3% (1/3)</td>
</tr>
<tr>
<td>Royal jelly</td>
<td>50·0% (1/2)</td>
<td>50·0% (1/2)</td>
<td>50·0% (1/2)</td>
</tr>
<tr>
<td>Brood food</td>
<td>80·0% (4/5)</td>
<td>80·0% (4/5)</td>
<td>60·0% (3/5)</td>
</tr>
<tr>
<td>Honeybees</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worker adults</td>
<td>100·0% (10/10)</td>
<td>90·0% (9/10)</td>
<td>90·0% (9/10)</td>
</tr>
<tr>
<td>Worker larvae</td>
<td>100·0% (11/11)</td>
<td>81·8% (9/11)</td>
<td>81·8% (9/11)</td>
</tr>
</tbody>
</table>
(87.2 %) was much higher than that from the ELISA method (20.2–23.8 %). In our studies, all tested samples that had viral capsid proteins as detected by ELISA were positive by RT-PCR, but most bees without capsid proteins tested positive by RT-PCR. Some samples with very high KBV RNA levels as detected by RT-PCR were negative by ELISA. The detection of low levels of viral-capsid proteins by ELISA may indicate that these healthy-appearing bees have low levels of viral infection. Alternatively, KBV may potentially be latent or persistent in a bee as a viral genome with extremely low levels of viral-capsid proteins.

We hypothesized that bee viruses (KBV and SBV) can be vectored by varroa mites and transmitted by both vertical- and/or horizontal-transmission routes among honeybees (Fig. 5). Anderson (1985) suggested that KBV could be transmitted by other means, as KBV was reported in Canada before the introduction of varroa mites to that country. Our results demonstrated that bee viruses (KBV and SBV) could potentially be transmitted either vertically from the queen to the eggs via transovarial transmission, or horizontally from workers to larvae or other bees via food sources containing glandular secretions. KBV and SBV were detected in both queens and eggs by RT-PCR, which indicates a route of transovarial transmission. Previously, Hung (2000) detected KBV in the faecal materials of worker and queen bees. In his experiment, queens were confined individually in separate Petri dishes. The faecal material was collected from each queen with a micropipette as soon as they defecated. In this experiment, one out of three queen excreta was positive by RT-PCR, suggesting that KBV was present in queens and that other bees could become infected when cleaning the hive.

Bee viruses (KBV and SBV) can potentially be transmitted horizontally via worker secretions. Our study demonstrated that bee viruses (KBV and SBV) were detected in worker bees, brood food, honey, pollen and royal jelly. These bee viruses may be transmitted from worker bees to larvae or to other adult bees (queen, other workers or drones) via food resources in the colony. It is also possible that bee viruses might be transmitted among colonies by feeding bees the honey or pollen gathered from diseased colonies (Fig. 4). This is a practice used by some bee-keepers to help colonies survive during times of low flowering. Also, dead or weakened colonies can be raided by worker bees from other colonies and honey and pollen taken back to their colonies. The importance of these food sources in viral transmission between colonies needs to be defined. Support for food being a route of transmission comes from studies done by Bailey (1969), who injected SBV preparations into adult bees and fed SBV to larvae, and then detected viruses in different tissues (head, abdomen, midgut and hypopharyngeal glands) by immunodiffusion. This experiment demonstrated virus in the hypopharyngeal glands of bees that had been injected with or fed SBV. The results also showed that more SBV accumulated in the heads of infected bees than in

Fig. 4. Detection of the RNAs of KBV and SBV and capsid proteins of KBV in varroa mites. (a) Both KBV and SBV RNAs were detected in samples with different numbers of mites (one, two, four or 12) by RT-PCR. N, No-template negative control for RT-PCR. (b) KBV capsid proteins were detected by ELISA in varroa mites (n = 9, 12 mites in each sample) and mite saliva collected via mite feeding on an artificial membrane (n = 2, each sample was collected from 20–25 mites over 24 h).

Fig. 5. Potential multiple infection routes for bee viruses (KBV and SBV). KBV and SBV may be transmitted from queens to eggs via vertical transmission, or from workers to larvae or other bees via secretions. Varroa mites may also transmit viruses to bees during feeding.
other regions. As pointed out by Bailey et al. (1964), adult bees detect and remove larvae with sacbrood within a day or two after the larvae die, while the virus is still infectious. Therefore, SBV is probably transmitted to the adults, as they can be infected by ingesting parts of dead larvae (especially ecdysial fluid). In turn, they resume feeding the larvae with secretions from their hypopharyngeal glands, thereby spreading the virus to other bees.

KBV and SBV can be detected simultaneously in an individual honeybee. Both viral RNAs could be amplified in the different developmental stages of bees, such as eggs, larvae, pupae and adults of queens, workers and drones. About half of the bee samples (69/139) had both KBV and SBV in the same bee, suggesting that these viruses could infect the same honeybee simultaneously, with both viruses being detected at high levels. Based upon serological tests, Anderson & Gibbs (1988) reported simultaneous, unapparent infections of KBV, SBV and Black queen cell virus (BQCV). When activated, KBV was thought to suppress the replication of SBV and BQCV. However, we did not observe this. In addition, Dall (1985) reported that there was no instance of mixed infection of KBV and SBV in bees in Australia. The reason for this discrepancy may lie in the difference of sensitivity of the virus-detection methods.

Varroa mites can be vectors for KBV and SBV. The world-wide spread of varroa mites in honeybee colonies has had a significant influence on viral infections in bees (Bakonyi et al., 2002). In Britain, before the invasion of the varroa mite, Acute bee paralysis virus (ABPV) never caused bee mortality whereas, after the invasion, bee mortality increased due to ABPV (Allen et al., 1986; Batuev, 1979). Allen et al. (1986) and Batuev (1979) suggested that varroa was associated with ABPV. By using immunodiffusion tests, Ball & Allen (1988) compared the prevalence of bee viruses in dead-bee samples from colonies infested with varroa mites with bees in uninfested colonies. The results demonstrated that ABPV titres were significantly higher in bee samples with varroa mites. They suggested that ABPV was the primary cause of adult bee mortality in honeybee colonies infested severely with Varroa jacobsoni. The authors suggested that the varroa mite potentially activated ABPV replication in adult bees by its feeding behaviour and potentially transmitted the virus from adult bees to pupae (Ball, 1983; Ball & Allen, 1988). In a review, Bailey & Ball (1991) suggested that the possibility that varroa mites may transmit KBV in the same manner as they do ABPV. Hung & Shimanuki (1999) and Hung et al. (2000) detected KBV in varroa mites by using RT-PCR. In our study, we have demonstrated that KBV and SBV can be detected simultaneously in varroa mites. Moreover, our detection of KBV capsid proteins in varroa-mite saliva suggests that varroa mites may potentially vector KBV via their saliva. Therefore, varroa mites may transmit viruses to bees during feeding. Also, RNAs of KBV and SBV can be detected in mites, even in single mites, indicating that the mites may also be infected with the virus. The intensity of this reaction suggested that the virus was present at high levels and was not just found in mouthparts or the digestive tract.

More recently, increasing knowledge of the interactions between honeybee viruses and parasitic mites (varroa mites and tracheal mites) has led to the suggestion that interactions may underlie honeybee mortality and colony collapse (Allen et al., 1986; Batuev, 1979; Brodsgaard et al., 2000; Korpela et al., 1992). To date, this relationship between the mite infestation and viral infection is not clearly understood. Also, there is little support for an increased SBV infection with varroa-mite infestation in adult bees. Our research has provided direct evidence for the role of varroa in vectoring bee viruses (KBV and SBV). In addition, we are testing the role of varroa mites in inducing viral infections in bees, and examining the interaction of these viruses and mite tissues.

ACKNOWLEDGEMENTS

We would like to thank Dr Xiaolong Yang for providing the mite saliva and Dr Joachim de Miranda for advice and help in recombinant protein expression. We thank Dr Scott Camazine for his great help in the initiation of the project and Owen Thompson for technical assistance. We are grateful to Dr Don Stoltz for providing the anti-VP4 antibody. We also thank Drs Craig Cameron and Fred Gildow for critical review of the manuscript. This work was supported by a grant from the Pennsylvania Department of Agriculture.

REFERENCES


Transmission routes of honeybee viruses


