

Short communication

Detection of viral sequences in semen of honeybees (*Apis mellifera*): Evidence for vertical transmission of viruses through drones

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Received 14 December 2005; accepted 2 March 2006

Available online 21 April 2006

Abstract

Honeybees (*Apis mellifera*) can be attacked by many eukaryotic parasites, and bacterial as well as viral pathogens. Especially in combination with the ectoparasitic mite *Varroa destructor*, viral honeybee diseases are becoming a major problem in apiculture, causing economic losses worldwide. Several horizontal transmission routes are described for some honeybee viruses. Here, we report for the first time the detection of viral sequences in semen of honeybee drones suggesting mating as another horizontal and/or vertical route of virus transmission. Since artificial insemination and controlled mating is widely used in honeybee breeding, the impact of our findings for disease transmission is discussed.

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Keywords: DWV; ABPV; Honeybee virus; Vertical transmission

Honeybees (*Apis mellifera*) are known and much valued as honey producers since several thousands of years. However, the economic value of honey production plays a minor role compared to the economic value of honeybees as pollinators of crops and fruit (Morse and Calderon, 2000). Considering their role in pollination, honeybees are among the most important productive livestock and indispensable for the agricultural ecosystem. Livestock breeding is common in husbandry and aims at introducing genetic improvement into livestock populations. Breeding programs in apiculture try to select for certain beneficial traits like, e.g., gentleness of the bees, honey productivity, and disease resistance. The latter is especially important since honeybees can be infected during any life stage by many bacterial and viral pathogens and can also be infested by several parasites. It is conceivable that these honeybee diseases have a direct effect on honeybee health and the performance of bee colonies and, hence, that they also affect the profitability of apiculture and agriculture.

Among the bee diseases, those caused by viral pathogens are the least understood. The most common virus infections are caused by acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), sacbrood virus (SBV), deformed wing virus (DWV), and Kashmir bee virus (KBV) (Allen and Ball, 1996; Tentcheva et al., 2004). With two exceptions (filamentous virus and *Apis* iridescent virus) all viruses identified in the honeybee so far are positive stranded picorna-like RNA-viruses grouped together with other invertebrate viruses either into the floating genus *Iflavirus* (SBV, DWV), as yet unassigned to any virus family, or into the family *Dicistroviridae* (ABPV, BQCV, KBV) (Christian et al., 2002a,b). The CBPV possesses an RNA genome but is not picorna-like (Bailey, 1976; Overton et al., 1982). Research in the area of honeybee viruses has long been hampered by the fact that (i) most viral infections seem to persist as inapparent infections or at least do not cause any symptoms recognized by the beekeeper (Bailey, 1976; Bailey and Ball, 1991 and (ii) genomic sequence data enabling molecular studies were not available until recently (de Miranda et al., 2004; Gosh et al., 1999; Govan et al., 2000; Leat et al., 2000). Therefore, little

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is known about the molecular pathogenesis and disease transmission of virus infections of honeybees.

ABPV has been detected in the thoracic salivary glands of adult bees and virus transmission to larvae by feeding was demonstrated (Bailey and Ball, 1991). For SBV, virus accumulation in the hypopharyngeal glands of adult bees and transmission through feeding have been reported (Bailey, 1969). A most recent study demonstrated SBV and KBV RNAs not only in larval food implicating horizontal transmission but also in eggs and queens suggesting a route of transovarial transmission for both viruses (Shen et al., 2005a). For DWV, transmission through the ectoparasitic mite *Varroa destructor* is well established and correlates with the occurrence of characteristic malformations like crippled wings (Ball, 1989; Bowen-Walker et al., 1999; Nordström, 2003; Yue and Genersch, 2005). In addition, detection of DWV sequences in larval food indicated feeding as a possible transmission route (Yue and Genersch, 2005). Recently, detection of DWV-sequences in laid eggs was reported suggesting also vertical transmission of DWV (Chen et al., 2005a).

Artificial insemination (AI) is extensively used in livestock breeding and dissemination of viral pathogens via semen (for review: Dejuq and Jegou, 2001) are of primary concern to breeders and regulatory authorities in countries where AI is widely practised. Since AI of virgin honeybee queens with pooled semen of several drones from selected colonies is a common method in honeybee breeding, the possibility of vertical transmission of honeybee viruses (Chen et al., 2005a; Shen et al., 2005a) prompted us to analyze whether or not honeybee semen obtained from drones do also contain viruses.

Drones were collected from honeybee colonies (*Apis mellifera*) selected for drone rearing in the course of the institute's breeding programs. Semen was collected from sexually mature honeybee drones. Drones were stimulated to ejaculate by pressing on the thorax until the cream-colored semen and a white mucus plug were released on the end of the penis. Under a dissecting microscope, 10X, the semen was collected without mucus into a syringe, designed by Schley (1982). Thirty-four seemingly healthy drones originating from eight different colonies showing no overt signs of viral infections were analyzed. Since only a small quantity, <1 µl, of semen can be collected from each drone, the semen of each of four or five drones was pooled ($n = 8$,

representing the eight colonies) to obtain a volume of around 3 µl for extraction of total RNA (RNeasy™, Qiagen). In some cases, corresponding semen collected from other drones was used for AI of virgin queens. Colonies founded by these queens were checked for overt signs of DWV- and ABPV-infection every second week in the season following AI. The colonies were apparently healthy without clinical symptoms of DWV- or ABPV-infection and developed normally.

Total RNA from semen was analyzed for the presence of viral sequences following sensitive and specific RT-PCR protocols for the detection of DWV (Genersch, 2005), ABPV (Bakonyi et al., 2002), and KBV (Stoltz et al., 1995). For detection of SBV, a primer pair was selected using MacVector 6.5 software, allowing establishing of multiplex RT-PCR detection of DWV and SBV. One-step-RT-PCR™ (Qiagen) reactions were performed with incubations for 30 min at 50 °C, 15 min at 95 °C followed by 35 cycles with 30 s at 94 °C, 30 s at the appropriate annealing temperature (see Table 1), 30 s at 72 °C followed by a final elongation step for 10 min at 72 °C. All pooled semen samples tested positive for ABPV, 50% contained also detectable levels of DWV RNA, demonstrating coinfection of these colonies with both viruses. No sequences of KBV and SBV were found (Fig. 1). The electrophoretic mobility of the RT-PCR amplicons (Fig. 1) correlated with their expected sizes (Table 1). The specificity of the amplicons was further verified by directly sequencing the RT-PCR products (Medigenomix) from the PCR reaction (ABPV) or after gel extraction (QIAquick™ Gel Extraction Kit, Qiagen) of the specific band (SBV, DWV).

In our study, we demonstrated for the first time sequences of DWV and ABPV being present in semen of honeybee drones indicating mating as route of virus transmission. All DWV-positive samples also contained ABPV and, therefore, the transmission of more than one virus through semen is possible. The presence of viruses in semen is demonstrated for many viruses and is a possible route of horizontal as well as vertical transmission in mammalian species (for review: Dejuq and Jegou, 2001). Our results now indicate that the same might be true for honeybees and, therefore, for invertebrates. Since honeybee queens mate with up to 28 drones (Kraus et al., 2005) the chance to catch virus-containing semen is not negligible even when only a minor proportion of drones are transmitting virus.

Table 1
Primers selected for RT-PCR analysis of KBV, ABPV, DWV, and SBV

Virus	Primer sequence	Length of amplicon (bp)	Annealing temperature	Reference
KBV	5'-GATGAACGTCGACCTATTGA-3' 5'-TGTGGGTTGGCTATGAGTCA-3'	414	50.5	Stoltz et al. (1995)
ABPV	5'-CATATGGCGAGCCACTATG-3' 5'-CCACTTCCACACAACCTATCG-3'	398	49.5	Bakonyi et al. (2002)
DWV	5'-CCTGCTAATCAACAAGGACCTGG-3' 5'-CAGAACCAATGTCTAACGCTAACCC-3'	355	52.0	Genersch (2005)
SBV	5'-GTGGCAGTGTGAGATAATCC-3' 5'-GTCAGAGAATGCGTAGTTCC-3'	816	52.0	Designed during this study

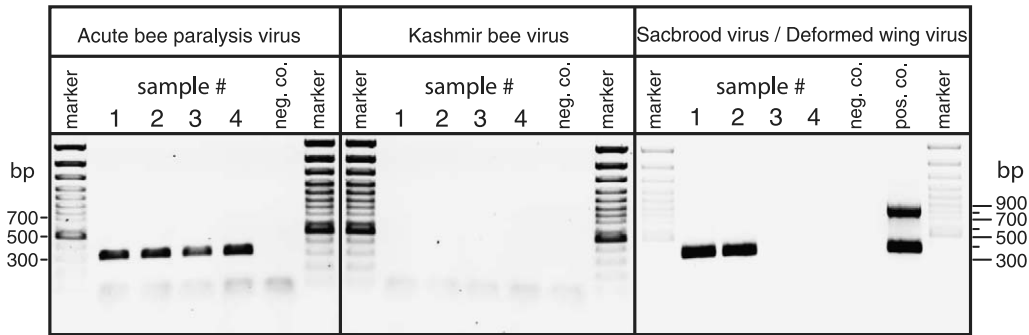


Fig. 1. Detection of viral sequences in semen collected from drones of *Apis mellifera*. RT-PCR analysis of acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), sacbrood virus (SBV), and deformed wing virus (DWV) in pooled semen samples (#1–#4) of apparently healthy drones generated specific amplicons for ABPV (398 bp) and DWV (355 bp). Representative results of four (#1–#4) out of eight samples as well as negative (neg. co.) and positive controls (pos. co.) are shown.

Virus-containing semen, therefore, is one possible source of virus-positive eggs (Chen et al., 2005a; Shen et al., 2005a) and also virus-positive queens (Chen et al., 2005b). Further studies are necessary to analyze how virus is transmitted into eggs during fertilization.

Surprisingly, the frequency of virus detection in semen does not correlate with the frequency of virus detection in bees. While 100% of adult bees in Germany were found to be DWV-positive (Yue and Genersch, 2005), only 50% of pooled semen samples did contain viral sequences indicating that virus transmission through semen is indeed only one of several transmission routes. On the other hand, all semen samples were positive for ABPV sequences although we rarely found ABPV in adult worker bees (data not shown). This would suggest that ABPV-containing semen not necessarily results in infected offspring.

However, although so far nothing is known about the impact this transmission route may have on bee health, we do not expect vertical transmission of viruses through sperm being a threat to beekeeping since most virus infections do not cause overt signs of disease (Bailey, 1976; Bailey and Ball, 1991). In this respect, it is important to note that no overt signs of virus infection were observed in the colonies founded by queens artificially inseminated with semen originating from brothers of drones from which virus-positive semen was collected. Obviously, the co-evolution of bees and viruses has resulted in a balance allowing both partners to survive and, therefore, “traditional” transmission routes are unlikely to cause severe damage to colonies. This picture of mainly benign virus infections in honeybees dramatically changed only when *V. destructor* entered the stage and introduced virus injection as a new route of transmission. The term Parasitic Mite Syndrome describes a complex of clinical symptoms mainly due to mite-dependent virus transmission or activation (Ball, 1983, 1989; Hung et al., 1996, 1995; Shimanuki et al., 1994). The combination of *V. destructor* infestation and different virus infections which can be either transmitted or activated by the mite (Bailey and Ball, 1991; Bowen-Walker et al., 1999; Shen et al., 2005b; Yue and Genersch, 2005) resulted in viral diseases of honeybees becoming a major problem in

apiculture, causing serious economic losses worldwide (Brodsgaard et al., 2000).

Yet, at the time being we cannot finally rule out detrimental effects of viral transmission through sperm since nothing is known so far about the amount of virus necessary in the queen’s spermatheca to allow horizontal infection of the queen or vertical transmission by infecting eggs or about any virus threshold sufficient to cause damage.

In conclusion, we demonstrated sequences of DWV and ABPV in sperm of apparently healthy drones and, hence, presented evidence for the possibility of vertical transmission of honeybee viruses. Further studies are necessary to unravel the possible horizontal and vertical transmission of honeybee viruses via semen and to analyze the impact of virus infections transmitted vertically via sperm and/or eggs, horizontally via, e.g., feeding or through *V. destructor* as vector.

Acknowledgments

The project 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.

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