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Detection of a honeybee iflavirus with intermediate characteristics between kakugo virus and deformed wing virus

Valentina Terio, Vito Martella, Michele Camero, Nicola Decaro, Gabriella Testini, Elisabetta Bonerba, Giuseppina Tantillo, Canio Buonavoglia

Department of Animal Health and Well-being, Faculty of Veterinary Medicine of Bari, Valenzano, Bari, Italy

SUMMARY

Iflavirus RNA was detected in honeybee colonies displaying unduly aggressive behavior and with no evidence of morphological alterations. Sequence analysis of the RNA-dependent RNA polymerase (RdRp) revealed that the iflavirus strain was more similar (>99% aa) to Deformed Wing Virus (DWV), that has been associated with morphological alterations in bees, rather than to the newly-described Kakugo Virus (KV) (about 95% aa), that has been associated with increased aggressiveness. Therefore, the iflavirus strain detected in the Italian hives genetically resembled DWV but was apparently associated with a KV-like phenotype. RT-PCR detected the iflavirus RNA in the abdomen of the workers, and only in one case was the virus detected in the head. No viral RNA was detected in the drones, a pattern of virus distribution across the honeybee casts that is in apparent conflict with the higher rates of infestation of drones by the mite *Varroa distructor*. The identification of a virus with apparently intermediate features between DWV and KV open new perspectives on the patho-biological role of iflaviruses in honeybees.

KEY WORDS: Honeybee, Kakugo-virus, Deformed wing virus

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INTRODUCTION

The European honeybee *Apis mellifera* is a eusocial insect, living in families encompassing thousand individuals. Apiculture is an important economic resource in several countries for production of honey, wax, propolis and royal jelly.

The insects have a nervous system with reduced complexity. Several honeybee behavioral traits have been linked to a genetic terrain, that includes stinging (Collins *et al.,* 1982*;* Moritz *et al.,* 1987), foraging for pollen or nectar (Hellmich *et al.,* 1985*;* Robinson and Page, 1989), undertaking the removal of dead bees from the nest (Rothenbuhler 1964), and learning (Brandes 1991; Bhagavan *et al.,* 1994).

The attacking behavior of guard bees is self-sacrificing and is therefore considered to be a typical altruistic behavior exhibited by the workers (Wilson 1975).

Thus, the honeybee is an attractive model for the study of altruistic aggressive behaviors (Fujiyuki *et al.* 2004). Quantitative trait locus analysis has been used to identify the loci related to aggressive worker behaviors (Hunt *et al.,* 1999; Hunt *et al.,* 1998*)*.

The genes responsible for the aggressive behaviors, however, have not yet been identified. Recently, Fujiyuki *et al.* (2004) studied the acquired aggressive behaviour of some bees against their natural enemy, the hornet *Vespa mandarinia japonica.* The aggressive behavior of the bees was not correlated to specific gene(s) but to the pres-

Corresponding author
Dr. Valentina Terio
Department of Veterinary Public Health
Faculty of Veterinary Medicine of Bari
Sp per Casamassima km 3 - 70010 Valenzano - Bari - Italy
E-mail: v.terio@libero.it

TABLE 1 - *Phenotypic characteristics associated with the honeybee iflavirus strains.*

	Virus strain		
Phenotype	*VT*	*KV*	*DWV*
SNC	○	●	
Abdomen	●		●
Aggressiveness	●	●	
Deformed wings			●

ence of a novel picorna-like virus, genus iflavirus, termed Kakugo (ready to attack). Kakugo Virus (KV) RNA has been detected in the brains of aggressive worker honeybees, suggesting a tight correlation between cerebral localization of KV and the aggressive behavior of honeybees.
KV is similar to another virus of honeybees, Deformed Wing Virus (DWV) transmitted via the bee mite, *Varroa destructor*. DWV infection occurs at the larval stage and is suggested to cause morphological deformity of wings in adult bees (DeJong *et al.,* 1982*;* Schatton-Gadelmayer and Engels, 1988). KV and DWV RNAs differ in a few amino nucleotides and deletions (Fujiyuki *et al.,* 2004) that are presumably responsible for the different virus-induced phenotypes in bees (Table 1).
The aim of the present study was to investigate the presence of KV/DWV in the honeybees of five hives of Apulia and Basilicata, where aggressive behavior of honeybees had been observed.

MATERIALS AND METHODS

Collection of honeybees

The investigation was carried out on five hives from Apulia and Basilicata, where aggressive behavior of the colonies had been reported.
Increased aggressiveness by the honeybees was presumed on the basis of repeated episodes of attack to humans in the absence of apparent dangers for the colony, such as close proximity and/or disturbance to the hives by workers. All the beehives were infested by the mite *Varroa destructor*, that is currently endemic in honeybee colonies in Southern Italy. Morphological alterations such as abdomen, thorax or wing deformity were not reported in the hives.
Between March and June 2004, for each family, 5 drones, 5 nurse bees, 5 attackers and 5 foragers were collected. Drones were gathered before leaving the cells. The insects were collected using the classical smoking-based technique. The insects were kept in glass vessels, narcotized with ethyl acetate and stored at -80°C before use.

RNA extraction

The heads and abdomens of 100 insects were dissected. For each honeybee typology 3 heads and 2 brains were examined. To collect the brain tissues, the head of the insects was stuck on a slide, and the occipital skull-cup was removed with a sterile noodle.
The brain was sucked up by a flame-modified pipette connected to a vacuum source. The heads in toto and the abdomens of the other honeybees were crushed with liquid nitrogen. Total RNA was extracted by the RNasy mini Kit (Qiagen, GmbH, Germany).

Polymerase Chain Reaction (PCR)

RNA was treated with RNAse-free water and then reverse transcribed with Superscript II one-step RT-PCR kit (Invitrogen Ltd, Paisley, UK). PCR was performed with gene specific primers 8388-8415 and 8745-8765 (Fujiyuki *et al.* 2004). In order to obtain additional sequence information, an additional primer, ValR, spanning from nt 10110 to 10135, was selected (5'-ATACTAAAATTAGGACGCATTACCA) (Figure 1). A fragment of about 1747 bp, encompassing the RNA-dependent RNA polymerase (RdRp) gene and the 3' untranslated region (UTR) was amplified with primers 8388-8415 and ValR by reverse transcription at 50°C for 45 min, and subsequent DNA amplification for 30 cycles at 94°C for 2 min, 62°C for 1 min and 68°C for 1 min. The PCR-generated DNA was visualized in 2% TAE agarose gel after staining with ethidium bromide. KV-positive bee tissue samples were kindly supplied by Dr. Tomoko Fujiyuki, Laboratory of Physiological Chemistry, Department of Biological Sciences, Graduate School of Science, University of Tokyo.

Sequence analysis

Four randomly selected PCR fragments, 378 bp, obtained with primers 8388-8415 and 8745-8765,

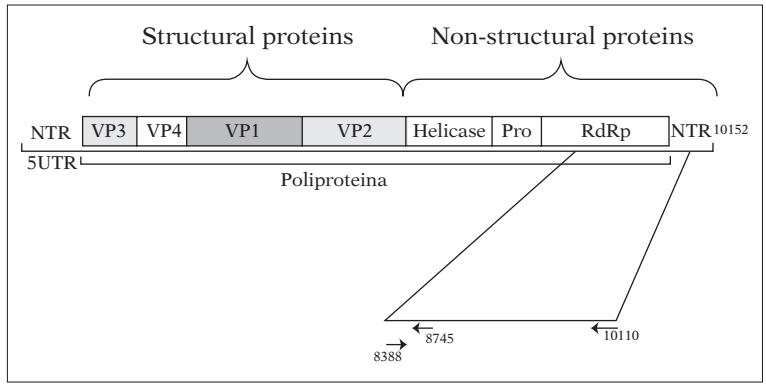

FIGURE 1 - *Schematic representation of the genome of Kakugo iflavirus. Primer position is shown by arrows.*

each representative of a different hive (VT-04-1, VT-04-2, VT-04-3, VT-04-4), were sequenced. Sequence analyses was performed after purification of the PCR product on Ultrafree DA Columns (Amicon Millipore, Bedford, USA) using an ABI-PRISM 377 (Applera Italia, Monza). The RdRp-encoding genome fragment of sample VT-04-02, generated with primers 8388-8415 and ValR, was sequenced after cloning with TOPO XL PCR Cloning kit (Invitrogen Ltd, Paisley, UK).
Sequences are freely available upon request.

RESULTS

By PCR with primers 8388-8415 and 8745-8765, 75 samples were found to be positive (the abdomens of nurse bees, attackers and foragers) while 25 samples were negative (the abdomens of drones). With the only exception of a forager bee of hive A, all the heads were found to be negative.
Samples VT-04-1, VT-04-2, VT-04-3 and VT-04-4 were found to be highly similar to each other (about 100% nt). Samples VT-04-2 (referred from here on as VT) was considered as prototype and the sequence of a large fragment at the 3' end of the viral genome (about 1747 bp), encompassing the RdRp, was determined. Strain VT was more similar to the DWV prototypes rather than to the KV prototype (Table 2).
The iflavirus identified in the hives from Apulia and Basilicata were found to have unusual phenotypic features, apparently intermediate between the prototype KV and the prototypes DWV (Table 1).

TABLE 2 - *Nucleotide (left side) and amino acid (right side) comparison of the iflavirus strain identified in Italy with other iflaviruses. The matrix of comparison was inferred on a fragment of about 1750 bp.*

	Viral strain					
Strain/Country/Year	*PA*	*DWI*	*DW*	*F*	*VT*	*KV VDV1*
PA/USA/2003	●	100	99.77	99.32	98.65	95.71
DWV I/Italy (Brescia)/2002	99.33	●	99.77	99.32	98.65	95.71
DWV F/France/2003	99.41	99.49	●	99.55	98.87	95.94
VT/Italy/Basilicata/2004	98.82	98.45	98.67	●	98.65	95.71
KV/Japan/2004	95.05	94.82	94.89	94.82	●	95.71
VDV1/Holland/2003	84.46	84.31	84.68	84.31	85.12	●

GenBank accession numbers: DWV I (AJ489744); DWV F (AY224602); PA (AY292384); KV 1 (AB070959); VDV1 (AY251269).

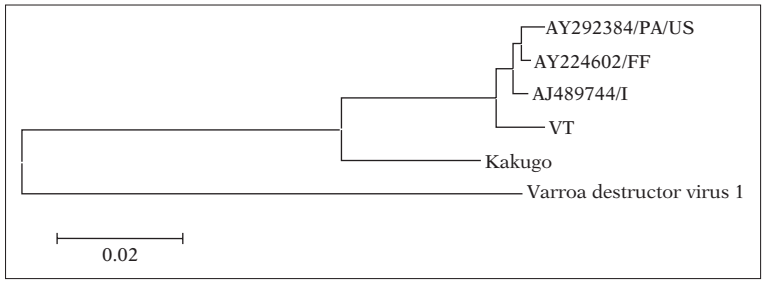

FIGURE 2 - *Phylogenetic analysis of the RdRp displaying the relationships among iflaviruses. The tree was inferred on the nucleotide alignment and it is rooted on strain VDV1.*

DISCUSSION

An iflavirus strain was detected in Italian honeybees by RT-PCR. To our knowledge, this is not the first report describing the presence of honeybee iflaviruses in Italy, as the sequence of a DWV-like iflavirus, strain DWV I, detected in honeybees affected by wings deformity in hives from Northern Italy, has been already registered in GenBank (accession AJ489744).

However, unlike strain DWV I, strain VT was not associated with morphological alterations in the bees, but to unduly aggressive behavior, thus resembling more the patho-biological patterns displayed by KV (Fujiyuki *et al.,* 2004), rather than the disease induced by DWV. By contrast, while KV was detected only in the brain (Fujiyuki *et al.,* 2004), the VT-like strains were mostly detected in the abdomens of the workers and only in one case in the brain of a forager bee (Table 3).

Excluding the possibility of errors during sample collection, it is likely that the iflavirus variant described by Fujiyuki *et al.* (2004), associated with a behavior alteration of bees, possesses a higher tropism for nervous tissues, while the variant identified in this study has a lower tropism for

TABLE 3 - *Results of RT-PCR analysis for KV/DWV in honeybees.*

Results of RT-PCR								
Hives	*Bee typology*	*N° of insects examined*	*Heads*		*Brains*		*Abdomens*	
			+	-	+	-	+	-
A	Drones	5	0	3	0	2	0	5
	Nurse bees	5	0	3	0	2	5	0
	Guardians	5	0	3	0	2	5	0
	Foragers	5	0	3	1	1	5	0
B	Drones	5	0	3	0	2	0	5
	Nurse bees	5	0	3	0	2	5	0
	Guardians	5	0	3	0	2	5	0
	Foragers	5	0	3	0	2	5	0
C	Drones	5	0	3	0	2	0	5
	Nurse bees	5	0	3	0	2	5	0
	Guardians	5	0	3	0	2	5	0
	Foragers	5	0	3	0	2	5	0
D	Drones	5	0	3	0	2	0	5
	Nurse bees	5	0	3	0	2	5	0
	Guardians	5	0	3	0	2	5	0
	Foragers	5	0	3	0	2	5	0
E	Drones	5	0	3	0	2	0	5
	Nurse bees	5	0	3	0	2	5	0
	Guardians	5	0	3	0	2	5	0
	Foragers	5	0	3	0	2	5	0

the nervous tissues, thus being absent in the brain or being present at levels under the limits of sensitivity of the RT-PCR assay. Alternatively, the presence of the VT-like virus in the nervous system is transient but the virus-induced alterations are persistent. Such explanation would be consistent with the fact that in one case it was possible to identify the virus in the brain and that the bees of the hives examined were displaying a highly aggressive behavior, whereas they did not display any wing alterations that are usually associated with infection by DWV. The phenotypic features associated with the prototypes KV and DWV (symptoms, localization in the insect tissues) are different and allow for a clear distinction of two separate pathologies (Table 3). The genetic differences between the Japanese prototype KV and the DWV strains identified in Italy, France and USA consist in about 200 nt substitutions, deletions and insertions, i.e. less than <2% of the genome. Only 21 nt variations are effective and determine amino acids (aa) changes, with 7 aa substitutions occurring in a highly conserved structural domain (Fujiyuki *et al.,* 2004). Such genetic diversity is likely responsible for the phenotypic differences observed between KV and DWV. It is noteworthy that the iflavirus identified in the hives of Apulia and Basilicata seems to be associated with intermediate phenotypic characteristics between KV and DWV. However, the Italian prototype VT is genetically more related to the DWV prototypes rather than the KV prototype and therefore minimal mutations/deletions in the genome of bee iflaviruses would be involved in the phenotype change.

This hypothesis is extremely intriguing, as small genetic variations in iflavirus genome would be related to drastic patho-biological changes, in a similar fashion to what has been observed in other virus models.

Recently, a novel iflavirus, VDV1 (Varroa destructor virus 1), was identified in the bee mite *Varroa destructor* in the Netherlands. Strain VDV1 is genetically different from the other bee iflaviruses and has been shown to replicate in mites under experimental conditions.

Nevertheless, neither deformed wings nor unduly aggressive behaviors were reported from the hives infested by the infected mites, suggesting that VDV1 infects asymptomatically or does not infect the honeybees at all (Ongus *et al.,* 2004) and puzzling the comprehension of iflavirus evolution and ecology. An intriguing point that emerged during our investigation is that all the drones tested by RT-PCR were found to be virus-negative. The mite *Varroa distructor* usually shows a tenfold preference to reproduce in drone cells and self-limiting density-dependent mechanisms tend to stabilize the mite population in overcrowded drone cells (Martin and Medina, 2004). Accordingly, because of the preferential infestation by *Varroa*, drones would be expected to be more exposed to infection by the mite-vectored iflavirus and the interactions between mites and honeybees could heavily affect virus pathogenicity. Bowen-Walker *et al.,* (1999) found a positive correlation between increasing numbers of mites on individual bees and the incidence of morphological deformity and death by DWV infection. Therefore, if no selection against is made by virus-induced alterations, virus incidence in the drone population should be considerably higher than in workers.

By contrast, if over-exposure to virus infection is lethal for drone broods and/or pupae, only non-infected insects can reach the adult stage and therefore, the fact that only non-infected adult males were collected during our survey would be due to early mortality of infected drones. The hypothesis that infection by VT-like viruses is associated with early mortality of drones warrants more in-depth investigations.

The present study contributes to the knowledge of iflaviruses in honeybees. The presence/persistence of KV/DWV-like viruses in Italian honeybees requires extensive, ongoing surveillance, as changes there coulkd be changes in pathogenicity to the DWV-like disease characterized by severe malformations of the bees, or even to new diseases, by acquisition of a few nucleotide/amino acid mutations. Further investigations are required to understand the geographic distribution of KV/DWV-like viruses and to obtain an estimate of the burden of such viruses in apiculture.

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