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Short communication. Presence, quantification and phylogeny of Israeli acute paralysis virus of honeybees in Andalusia (Spain)

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Abstract

This study aimed to assess the possible relationship between the presence of Israeli acute paralysis virus (IAPV) of honeybees and disease symptoms development at the colony level, to describe the IAPV load in field colonies and to illustrate phylogenetic relationships between IAPV isolates in Andalusia (Spain). Presence and load of IAPV was studied in 96 colonies from all provinces in Andalusia. Epidemiological surveys were performed in all the colonies to assess their sanitary status. IAPV was found in 13.5% of the sampled colonies, and no association was observed between the presence of IAPV and disease symptoms at the colony level. An average IAPV load was established in $4.9 \cdot 10^5$ genome equivalent copies per bee. Phylogenetic analysis revealed that Andalusian isolates belong to a different lineage to a previously described isolate found in Valencia (2010). The results of this study will help us understand the epidemiology and effect of IAPV on Spanish colonies.

Additional key words: *Apis mellifera;* viral load; phylogenetic analysis; epidemiological survey.

Israeli acute paralysis virus (IAPV) of honeybees is a Dicistrovirus that was first described in 2004 in Israeli colonies that had suffered from heavy losses (Maori *et al.,* 2007). This virus gained prominence after a report in 2007 associated it with the colony collapse disorder (CCD) in the USA, where IAPV presence was considered a statistically significant marker of CCD (Cox-Foster *et al.,* 2007). IAPV has since been described in numerous countries such as China (Xun *et al.*, 2009), Canada and Australia (Palacios *et al.*, 2008), or Argentina (Reynaldi *et al.*, 2011). In Europe, IAPV has been found in France (Blanchard *et al.*, 2008) and more recently in Spain (Kukielka & Sánchez-Vizcaíno, 2010) and Poland (Pohorecka *et al.*, 2011). However, the high frequencies found in some countries (up to 41% in Argentina) and lack of obvious disease symptoms in most sampled colonies suggest that IAPV is a widespread virus that usually appears in covert infections, like most Dicistroviruses (De Miranda *et al.*, 2010). Criteria identification to differentiate covert from overt infections in honeybee colonies is essential to identify risk factors of honeybee diseases, including CCD, where viruses may play an important role. Viral load may influence the development of disease symptoms, thus studies that differentiate the virus load causing covert and overt infections at the colony level are required. Some studies have quantified IAPV loads in experimental infections (Maori *et al.*, 2007, 2009) and have observed high mortality upon inoculation and feeding with purified viral particles. However, none of these studies has assessed IAPV loads in field colonies (commercial hives) until recently (Martin *et al.*, 2012). Another explanation for the covert infection of IAPV is the virulence of the isolate. Most published isolates are low-virulent, as IAPV normally persists in the colony in covert infections, showing no obvious symptoms at the individual or colony level (De Miranda *et al.*, 2010). In Spain, only one sequence has been phylogenetically analysed from one healthy colony in Valencia (Kukielka & Sánchez-Vizcaíno, 2010) despite

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Abbreviations used: ABPV (Acute bee paralysis virus); CCD (colony collapse disorder); CERA (the Beekeeping Reference Centre of Andalusia); COAG (Coordination of Agricultural and Livestock Organisations); GEC (genome equivalent copies); IAPV (Israeli acute paralysis virus), IGR (intergenic region); KBV (Kashmir bee virus); ORF (open reading frame); PBS (phosphate-buffered saline); RT-PCR (reverse transcription polymerase chain reaction); RT-qPCR (real-time reverse transcription polymerase chain reaction).

a report of 18% IAPV frequency in 2006 (Garrido-Bailón *et al.*, 2010) and 13% and 25.7% in 2006 and 2007, respectively (Antúnez *et al.*, 2012). This Valencian IAPV isolate was surprisingly clustered with isolates from collapsed colonies from geographically far countries. Thus, new phylogenetic analyses of IAPV are required in Spain to confirm its phylogeny in relation to virulence as compared with other isolates from different countries.

This study aimed to: assess the possible relationship between IAPV presence of honeybees and disease symptoms at the colony level; describe IAPV load in commercial hives; and illustrate the phylogenetic relationships between IAPV isolates by focusing on Andalusia, an important Spanish beekeeping region.

This work studied Andalusia (south-east Spain), which ranks first in hive censuses and is the second most important Spanish honey-producing region. Technicians from the Coordination of Agricultural and Livestock Organisations (COAG) and the Beekeeping Reference Centre of Andalusia (CERA, University of Córdoba) sampled 96 honeybee colonies from all provinces of Andalusia in winter 2010/2011. The study was designed to describe frequencies of honey bee viruses in this autonomous community. Assuming the worse prevalence scenario (50%), sample size was calculated by considering hive censuses with an expected error of 10% and a 95% of confidence interval with WinEpiscope v2.0 (De Blas *et al.*, 2000). One hundred different aged adult bees (nurse bees, guard bees, foragers) were collected per colony and frozen immediately afterwards. Epidemiological data were also collected during sampling by surveys to assess sanitary conditions. No clear CCD symptoms, collapse nor severe winter losses were found in the sampled colonies. Thus the present study focused on the potential association of IAPV with colony weakening represented by colonies with worse health status. Worse health status indicators included presence of clear IAPV disease symptoms, such as paralysis, loss of hair or inability to fly, depopulation, kleptoparasitism of stronger neighbouring colonies, diagnosed disease potentially associated with immune depletion such as chalkbrood (Glinski & Buczek, 2003; Aronstein & Murray, 2010); and problems to control *Varroa destructor* – mite infestation might lead to immunosuppression (Yang & Cox-Foster, 2005), which may facilitate IAPV replication, as *Varroa destructor* has been described as an IAPV vector (Di Prisco *et al.*, 2011). Sanitary status data were collected from the epidemiological surveys conducted by technicians upon sampling. Colonies were classified as "healthy" or "weak" depending on the above-described symptoms appearing or not. Each analytical sample consisted of 50 bees, which were homogenized with 6 mL of sterile phosphate-buffered saline (PBS) using a mortar and pestle. RNA extraction was carried out using the column-based Nucleospin II Virus® kit (Macherey Nagel) following the manufacturer's instructions.

Samples were analysed for IAPV presence by amplifying virus-specific nucleic acid. One-step real-time reverse transcription polymerase chain reaction (RT-qPCR) was carried out using SYBR-Green dye. Primers targeting a 220-bp region of the gene ORF-2 were used (Palacios *et al.,* 2008), and amplification was done following the thermocycler protocol described by Palacios *et al.* (2008).

Absolute quantification of IAPV-positive samples was performed to determine their viral load. The ORF-2 fragment of IAPV was cloned into a PGemT® TA cloning vector (Promega) following the manufacturer's instructions. The standard curve was constructed with triplicates of serial dilutions of known amounts of plasmid DNA. The viral load results were expressed in genome equivalent copies (GEC) per bee (Gauthier *et al.*, 2007). One sample from Valencia, previously analysed for IAPV presence in 2010 (Kukielka & Sánchez-Vizcaíno, 2010), was included in the samples quantification with identical procedures because its viral load was not quantified in the previous study. This sample was also included in the phylogenetic analysis as it was the first IAPV isolate found in Spain.

After confirming IAPV presence, RNA samples were first re-amplified by conventional RT-PCR targeting of a 705-bp sequence, which contains the 3' end of ORF1 (including part of the RdRp polymerase), the intergenic region (IGR) (183-bp) and the 5' end of the ORF2 region -including part of viral protein 2 (VP2)- of the IAPV genome (Palacios *et al.,* 2008), and were then sequenced. This fragment contains a variable region considered adequate to perform the IAPV phylogenetic analyses, as the highly conserved nature of the RdRp region used in other bee virus phylogenetic analyses can produce cross-reactivity between close KBV and IAPV viruses (Palacios *et al.*, 2008). The resulting 705-bp sequences were utilised to construct a phylogeny of the identified isolates. Complete sequences of the amplified region were aligned with other IAPV sequences from different countries using MEGA 4 (Tamura *et al.,* 2007) (Table 1). A phylogenetic

Table 1. Israeli acute paralysis virus (IAPV) sequences used to calculate the phylogenetic tree, shown in order of publication. Acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) sequences were included as the out-group

GenBank accession No.	Country	Virus	Reference
AF150629	UK	ABPV	Govan *et al.*, 2000
AY275710	USA	KBV	de Miranda *et al.*, 2004
EU122347	Australia	IAPV	Cox-Foster *et al.*, 2007
EU122348	Australia	IAPV	Cox-Foster *et al.*, 2007
EU122349	Australia	IAPV	Cox-Foster *et al.*, 2007
EU122350	USA	IAPV	Cox-Foster *et al.*, 2007
EU122356	USA	IAPV	Cox-Foster *et al.*, 2007
EU122357	USA	IAPV	Cox-Foster *et al.*, 2007
EU122358	USA	IAPV	Cox-Foster *et al.*, 2007
EU122361	USA	IAPV	Cox-Foster *et al.*, 2007
EU122362	USA	IAPV	Cox-Foster *et al.*, 2007
EU436427	Canada	IAPV	Palacios *et al.*, 2008
EU436432	USA	IAPV	Palacios *et al.*, 2008
EU436443	USA	IAPV	Palacios *et al.*, 2008
EU436446	Australia	IAPV	Palacios *et al.*, 2008
EU436447	Australia	IAPV	Palacios *et al.*, 2008
EU436448	Australia	IAPV	Palacios *et al.*, 2008
EU436455	Israel	IAPV	Palacios *et al.*, 2008
EU436456	Australia	IAPV	Palacios *et al.*, 2008
EU604006	France	IAPV	Blanchard *et al.*, 2008
EU604007	France	IAPV	Blanchard *et al.*, 2008
EU604008	France	IAPV	Blanchard *et al.*, 2008
EU604009	France	IAPV	Blanchard *et al.*, 2008
EU604010	France	IAPV	Blanchard *et al.*, 2008
FJ754324	China	IAPV	Xun *et al.*, 2009
EU218534	USA	IAPV	Chen Y., published in Genbank, 2009
FJ821506	Spain	IAPV	Kukielka & Sánchez-Vizcaíno, 2010
JQ435732-JQ435744	Spain	IAPV	Present study

tree was created by using the neighbour-joining test (Fig. 1).

The viral diagnosis results showed that IAPV was present in 13 of the 96 samples (13.5%) with $4.9 \cdot 10^5$ GEC/bee on average (minimum-maximum GEC/bee: $8.6 \cdot 10^3 – 1.2 \cdot 10^7$). The IAPV load of the Valencian sample was $1.43 \cdot 10^5$ GEC/bee. The Andalusian IAPV frequency results were similar to those previously obtained (Garrido-Bailón *et al.*, 2010) with IAPV present in 18 out of 100 samples from 33 Spanish provinces; and more recently, to those of a retrospective study (Antúnez *et al.,* 2012) describing IAPV frequencies of 13% and 25.7% in 2006 and 2007, respectively. Other than IAPV frequency in Andalusia, viral quantification showed that IAPV was present in substantial loads (up to 10^7 GEC/bee). Another study on adult honeybees at the colony level found viral loads of six different honeybee viruses in the order of 10^8 and 10^9 GEC/bee (Gauthier *et al.*, 2007). More recently, Martin *et al.* (2012) reported similar results on the Hawaiian islands with IAPV loads in the order of 10^7 GEC/bee in covert infections not associated with colony collapse, but with lower prevalences (only 3 colonies of 293 were infected with IAPV). Based on the recent description of the *V. destructor* mite as a transmitter of IAPV (Di Prisco *et al.*, 2011), future studies should also determine IAPV loads in pupae and *V. destructor* to better understand the biology of the virus. In this study, only the effect of IAPV presence and load was considered on weakening colonies in Andalusia. Future weakening colonies research should also contemplate other pathogens, the effect of co-infections and the host-pathogen-environment interaction. Given the IAPV frequency and load in Andalusia, these results suggest that IAPV presence is not an anecdotic finding in Andalusia.

According to the epidemiological surveys, 32 colonies were classified as “weak” and showed one or more above-described disease symptoms. No colony reported CCD or collapse. Eight of these weak colonies were positive to IAPV (25%), but IAPV presence was not

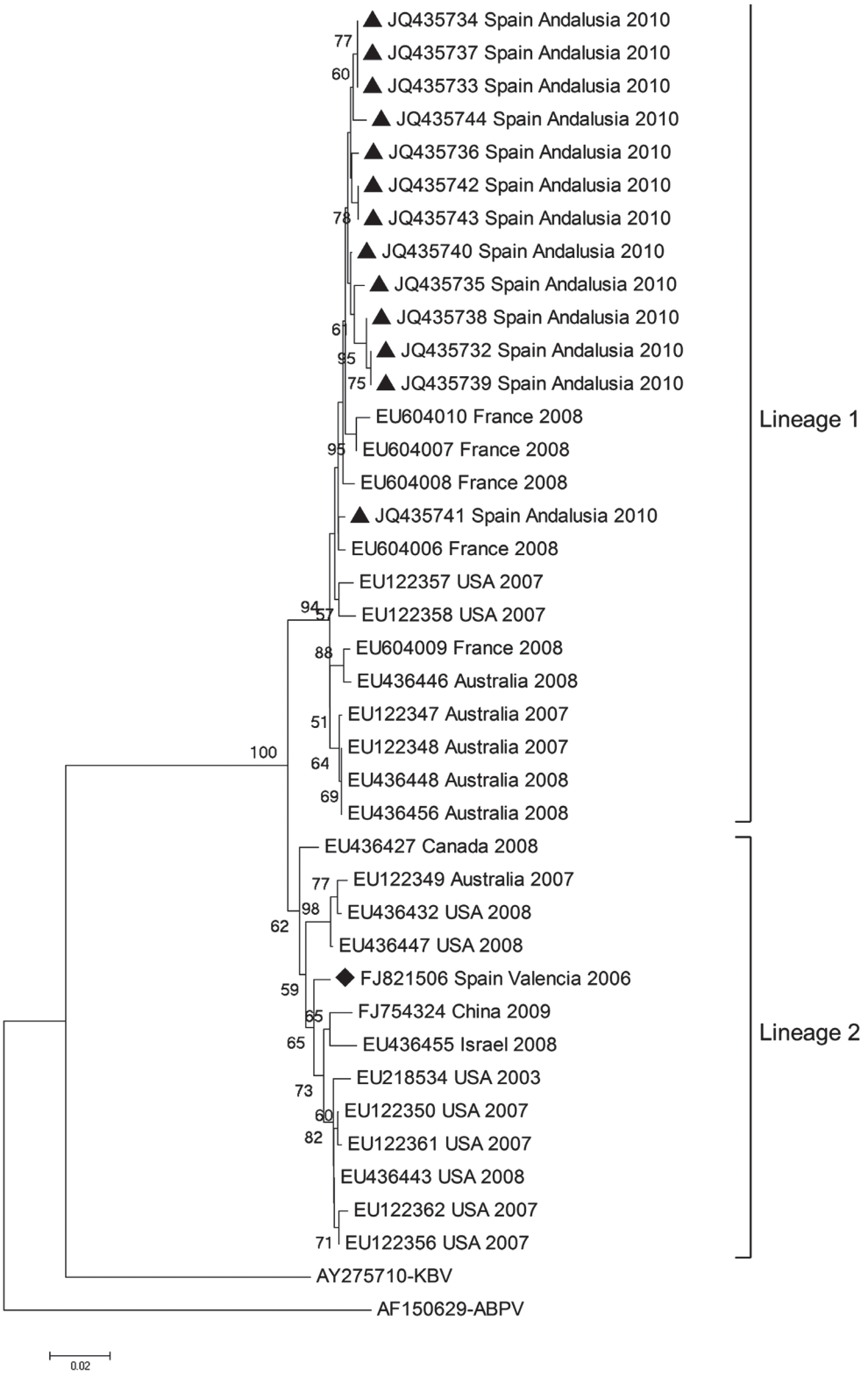

Figure 1. "Neighbour-joining" phylogenetic tree of IAPV sequences. Triangles indicate sequences from Andalusia and the diamond indicates the sequence from Valencia. Acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV) sequences were included as the out-group. Genbank accession numbers, sampling location and year are shown per sequence. The number of each node represents the bootstrap values as the result of 1,000 replicates. Bootstraps values of < 50% were omitted.

associated with weakening (χ^2 test, $p > 0.05$). No specific symptom contemplated in the epidemiological surveys was statistically associated with IAPV presence. Besides, the highest viral loads were present in both healthy and weak colonies, indicating that IAPV loads in the order of 10^7 GEC/bee do not produce obvious disease symptoms at the colony level themselves. However, it would be interesting to perform molecular and histopathological analyses of individual bees to describe the proportion of infected bees per colony as pools of bees were used, thus we cannot ensure that these viral loads do not produce clinical symptoms in individual bees. Future studies should consider this issue to establish the IAPV load baseline in healthy commercial hives.

The phylogenetic analysis revealed two main lineages of IAPV (Fig. 1). The first one grouped all the IAPV sequences from Andalusia in the present study with IAPV isolates mostly from colonies suffering severe winter losses in France. The Valencian isolate (Kukielka & Sánchez-Vizcaíno, 2010) was, however, grouped with the second lineage, which included isolates from collapsed colonies in USA, China, Australia, Israel and Canada. These results confirm the great genetic diversity of IAPV sequences identified in Spain and suggest at least two differentiated evolutionary IAPV lineages in Spain. Andalusian samples may relate more closely to French samples given geographical proximity since natural bee migration between Spain and France can occur in lower-altitude areas of the Pyrenees, near the Atlantic and Mediterranean coasts. Hence, colonies separated by approximately 1,000 kilometres, like those in Andalusia and the northern Spain, can come into contact when beehives are moved to pollinate crops during transhumance, a common practice in Spain where 30.2% (7,323 of 24,251) of colonies are transhumant (MAGRAMA, 2012). The Valencian isolate is phylogenetically divergent from the IAPV isolates described in Andalusia and relates instead to isolates from geographically remote regions. Trade is the most likely explanation for the spread of IAPV between countries; imports of IAPV-positive honeybees from Australia to the USA have been reported (Palacios *et al.,* 2008). The fact that IAPV isolates from healthy and symptomatic hives cluster together suggests that the symptoms development may be linked with viral replication activation and presence of high viral loads. This fact has been suggested (Ribière *et al.*, 2008), but is still to be verified in IAPV. Since IAPV has been found in many Spanish regions, further phylogenetic analyses are recommended in future studies on IAPV in honeybees. To confirm the results described herein, these future analyses should include the whole genome of the studied IAPV isolates.

Here the results suggest that IAPV is no sporadic finding in Andalusia because its frequency is 13.5% and IAPV presence does not significantly associate with disease symptoms presence at the colony level in this region. Therefore, we report IAPV loads in the order of up to 10^7 GEC/bee in healthy commercial hives, which indicates that presence of these viral loads is not indicative of overt infection at the colony level. Therefore, the development of obvious disease symptoms at this level must be produced by higher viral loads, probably in conjunction with other factors and especially in immunosuppressed colonies. Moreover, Andalusian IAPV isolates are phylogenetically distant from the Valencian isolate, suggesting that IAPV has at least two different evolutionary lineages in Spain. Spanish isolates from healthy colonies relate to isolates from colonies suffering depopulation or collapse in other countries, thus further investigation into isolates virulence in field samples is required. Indeed, phylogenetic analysis may help investigate relevant honeybee virus epidemiology aspects, such as their spread via trade routes, which must be considered when implementing control measures.

In conclusion, this study reports the IAPV frequency in an important Spanish beekeeping region, Andalusia, and lack of association between IAPV presence and disease symptoms at the colony level. It is also the first quantification of IAPV load in Spain and it has established phylogenetic similarities between Andalusian IAPV isolates and those mainly from France by identifying at least two different evolutionary IAPV lineages in Spain.

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References

Antúnez K, Anido M, Garrido-Bailón E, Botías C, Zunino P, Martínez-Salvador A, Martín-Hernández R, Higes M, 2012. Low prevalence of honeybee viruses in Spain during 2006 and 2007. Res Vet Sci 93(3): 1441-1445.

Aronstein KA, Murray KD, 2010. Chalkbrood disease in honey bees. J Invertebr Pathol 103(Suppl 1): S20-29.

Blanchard P, Schurr F, Celle O, Cougoule N, Drajnudel P, Thiery R, Faucon JP, Ribière M, 2008. First detection of Israeli acute paralysis virus (IAPV) in France, a dicistrovirus affecting honeybees (*Apis mellifera*). J Invertebr Pathol 99: 348-350.

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan PL, Briese T, Hornig M, Geiser DM *et al*., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318: 283-287.

De Blas N, Ortega C, Frankena K, Noordhuizen J, Thrusfield M, 2000. WinEpiscope version 2.0. University of Edinburgh.

De Miranda JR, Drebot M, Tyler S, Shen M, Cameron CE, Stoltz DB, Camazine SM, 2004. Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. J Gen Virol 85: 2263-2270.

De Miranda JR, Cordoni G, Budge G, 2010. The acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. J Invertebr Pathol 103(Suppl 1): S30-S47.

Di Prisco G, Pennacchio F, Caprio E, Boncristiani HF, Evans JD, Chen Y, 2011. *Varroa destructor* is an effective vector of Israeli acute paralysis virus in the honeybee, *Apis mellifera*. J Gen Virol 92: 151-155.

Garrido-Bailón E, Martín-Hernández R, Bernal J, Bernal JL, Martínez-Salvador A, Barrios L, Meana A, Higes M, 2010. Short communication. The detection of Israeli Acute Paralysis virus (IAPV), fipronil and imidacloprid in professional apiaries are not related with massive honey bee colony loss in Spain. Span J Agric Res 8: 658-661.

Gauthier L, Tentcheva D, Tournaire M, Dainat B, Cousserans F, Colin ME, Bergoin M, 2007. Viral load estimation in asymptomatic honey bee colonies using the quantitative RT-PCR technique. Apidologie 38: 426-435.

Glinski Z, Buczek K, 2003. Response of the *Apoidea* to fungal infections. Apiacta 38: 183-189.

Govan VA, Leat N, Allsopp M, Davison S, 2000. Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect-infecting RNA viruses. Virology 277: 457-463.

Kukielka D, Sánchez-Vizcaíno JM, 2010. Short communication. First detection of Israeli Acute Paralysis Virus (IAPV) in Spanish honeybees. Span J Agric Res 8: 308-311.

MAGRAMA, 2012. El sector de la miel en cifras. Principales indicadores económicos de 2011. Spanish Ministry of Agriculture, Food and Environment. Available in http://www.magrama.es/. [March 2012].

Maori E, Lavi S, Mozes-Koch R, Gantman Y, Peretz Y, Edelbaum O, Tanne E, Sela I, 2007. Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to intra- and inter-species recombination. J Gen Virol 88: 3428-3438.

Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, Sela I, 2009. IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. Insect Mol Biol 18(1): 55-60.

Martin SJ, Highfield AC, Brettel L, Villalobos EM, Budge GE, Powell M, Nikaido S, Schroeder DC, 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336(6086): 1304-1306.

Palacios G, Hui J, Quan PL, Kalkstein A, Honkavuori KS, Bussetti AV, Conlan S, Evans J, Chen YP, vanEngelsdorp D, *et al*., 2008. Genetic analysis of Israel acute paralysis virus: distinct clusters are circulating in the United States. J Virol 82: 6209-6217.

Pohorecka K, Zdanska D, Bober A, Skubida M, 2011. First detection of Israeli acute paralysis virus (IAPV) in Poland and phylogenetic analysis of the isolates. J Apicult Sci 55(2): 149-159.

Reynaldi FJ, Sguazza GH, Tizzano MA, Fuentealba N, Galosi CM, Pecoraro MR, 2011. First report of Israeli acute paralysis virus in asymptomatic hives of Argentina. Rev Argent Microbiol 43: 84-86.

Ribière M, Ball B, Aubert MFA, 2008. Natural history and geographical distribution of honey bee viruses. In: Virology and the honey bee (Aubert M, Ball B, Fries I, Moritz R, Milani N, Bernardinelli I, eds). European Commission, Brussels. pp: 15-84.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: molecular evolutionary genetics analysis software version 4.0. Mol Biol Evol 24: 1596-1599.

Xun Y, XueJiao L, JingHua C, Yi Z, RiChou H, 2009. First detection of Israel acute paralysis virus (IAPV) in *Apis mellifera* (Hymenoptera: Apidae) in China. Sociobiology 54: 95-100.

Yang Y, Cox-Foster DL, 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc Natl Acad Sci 102(21): 7470-7475.