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Short communication

Identification of Kashmir bee virus in France using a new RT-PCR method which distinguishes closely related viruses

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A B S T R A C T

A new RT-PCR protocol has been developed, avoiding potential misdiagnosis of Kashmir bee virus (KBV) linked to the use of KBV primers designed originally. The PCR assay validation was realised taking into account the analytical specificity and the PCR detection limit. KBV was detected in a bee sample collected in France from an apparently healthy apiary in 2012. The specificity of the primers was confirmed by sequencing the PCR product. This French sequence clustered into the KBV genotype by phylogenetic analysis, while previous French sequence isolates collected in 2002 belong to the IAPV genotype. These data represent the first detection of KBV in France.

1. Short communication

Kashmir bee virus (KBV) was discovered in 1974 as a contaminant in preparations of Apis iridescent virus extracted from *Apis cerana* (Bailey et al., 1976). It was isolated from *Apis mellifera* after experimental infection (Bailey and Woods, 1977). While KBV is serologically, biologically and genetically related to Acute bee paralysis virus (ABPV) (Anderson, 1991; Allen and Ball, 1995; Evans, 2001; de Miranda et al., 2004), the phylogenetic analysis confirms that KBV and ABPV are close but distinct viral species (de Miranda et al., 2004). A new member of the *Dicistroviridae* family, the Israeli acute paralysis virus (IAPV), closely related to KBV and ABPV, but genetically and serologically distinct, was also characterized (Maori et al., 2007). These three viruses are part of a complex of closely related viruses from the *Dicistroviridae* family, classified recently into the *Aparavirus* genus (King et al., 2011). They have a widespread prevalence in honey bee colonies and could be frequently implicated in honey bee colonies losses, especially when the colonies are infested with the parasitic mite *Varroa destructor* (de Miranda et al., 2010).

The impact of KBV infection on honey bee colonies is still poorly understood (Anderson, 1991; Hung et al., 1996). Molecular diagnosis of Kashmir bee virus infection was first developed by Stoltz et al. (1995) and often used to evaluate prevalence of KBV in different countries (Hung et al., 2000; Evans, 2001; Tentcheva et al., 2004; Siede et al., 2005; Nielsen et al., 2008; Blanchard et al., 2008; Al-Abbadi et al., 2010; Toplak et al., 2012; Cersini et al., 2013). However, it has previously been shown that primers described by Stoltz et al. (1995) were not specific for KBV and could also pick up IAPV, which led to the first description of IAPV in France (Blanchard et al., 2008). In fact, other KBV-like sequences were misclassified such as the Australian KBV-like sequences described by Hung et al. (2000) and the French KBV-like sequences described by Tentcheva et al. (2004) which were also more closely related to IAPV than to KBV (Blanchard et al., 2008). de Miranda et al. (2010) also pointed out that isolates from France, Australia, Jordan, Russia and South Korean were initially misclassified as KBV since, after phylogenetic analysis, they clustered into the IAPV genogroup. Real-time RT-PCR was also developed to assess the KBV prevalence, using TaqMan® technology (Ward et al., 2007) or SYBR® green technology (Antunez et al., 2012), resulting in a low prevalence in England and Spain (0.4%). However, the analytical specificity of the SYBR® green methods was not always demonstrated.

Carletto et al. (2010) described the development of multiplex PCR assays for the detection of 14 distinct bee pathogens including 7 viruses, 4 bacteria, and 3 fungi. For KBV

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detection, a primer pair was designed by using the "Fast-PCR" software according to alignments of KBV, ABPV and IAPV sequences. Primers were located in the polyprotein of the KBV sequence (reference GenBank no. AY275710) and named KBV-F2952: 5′-TATGCTGAACAACGCAAAGA-3′ and KBV-R3610: 5′-ACAACACGATGTCTGGGTTT-3′, producing a 659 bp amplicon. This primer set was used to amplify RNA from a KBV German isolate (Siede and Büchler, 2004). The purified PCR product was then sequenced in both orientations and shared 94.3% of identity with the KBV reference sequence (GenBank no. AY275710). This RNA sample was also subjected to PCR using the first KBV primers described by Stoltz et al. (1995). The obtained PCR product was sequenced. It showed 96% and 99.5% identity, with the KBV reference sequence and the German KBV sequence (GenBank no. AY787143), respectively. The RNA sample was also subjected to KBV PCR using primers described by Shen et al. (2005), giving the expected amplicon.

This KBV PCR assay was validated according to the AFNOR XP U47-600 standard as already described for chronic bee paralysis virus (CBPV) by Blanchard et al. (2012). Analytical specificity was assessed according to an *in silico* analysis on KBV primers by a Blast search on the NCBI genome database, and to the exclusivity test on cDNAs obtained from bee samples infected with other bee viruses. No significant similarity with KBV primers was found and no significant amplification was detected in cDNAs from ABPV, CBPV, IAPV and DWV, confirming the exclusive specificity. To assess the detection limit of the KBV PCR assay (DL_{PCR}), the pN2 plasmid (3.67 kb) was used, obtained by cloning the 659 bp PCR fragment into a pGEM®-T Easy vector (Promega). The DL_{PCR} was determined using three independent 2-fold dilution series of the 3.67 kb plasmid ranging from 2000 to 62.5 DNA copies in 2.5 μl of template, with 8 replicate reactions for each dilution series. The lowest number of nucleic acid targets detected in at least 95% of the 24 replicates was 250 DNA copies. The DL_{PCR} of KBV PCR was thus defined as 250 genome copies per reaction.

This new KBV PCR was further tested on 84 bee samples collected in France in 2012. 27 bee samples collected between February and April came from apiaries presenting winter losses while 57 bee samples collected between May to August came from apparently healthy apiaries. Sample preparation (pooled samples of 8 to 10 bees), RNA extraction and cDNA synthesis were performed as described previously (Blanchard et al., 2007). Molecular detections (ABPV, IAPV, DWV, BQCV, CBPV, SBV and KBV) were performed using primer pairs respectively described by Bakonyi et al. (2002), Cox-Foster et al. (2007), Blanchard et al. (2007), Grabensteiner et al. (2001) and from this study. Unexpectedly, KBV was detected only in one sample taken from an apparently healthy colony, while no other bee viruses tested were detected in this sample. ABPV, IAPV, DWV and BQCV were detected respectively in 14.3%, 3.6%, 48.8% and 58.3% of the samples. CBPV was detected in 3.6% of bee samples with viral loads in correlation with chronic bee paralysis clinical symptoms. Furthermore, detection of *Nosema* spp. was carried out following the OIE method (2008). The molecular discrimination of *Nosema apis* and of *Nosema ceranae* were performed as described by Carletto et al. (2013), following the protocol using two singleplex PCRs with primers described by Martín-Hernández et al. (2007). *N. ceranae* was identified in 15.5% of bee samples.

PCR product (659 bp) obtained from the KBV-positive sample (S2032) was sequenced in both orientations and compared to the KBV reference sequence (GenBank no. AY275710) and the German sequence as above, showing respectively 99% and 94% of identity. In order to confirm the identification of this KBV sequence, the sample was also subjected to KBV PCR using primers described by Stoltz et al. (1995). The resulting PCR product was purified and sequenced in both orientations. This sequence was used for the phylogenetic analysis to compare with ABPV, IAPV and KBV isolates from various geographical origins. After exclusion of the primers sequences, the nucleotide sequences reported in Table 1 were aligned by using the CLUSTAL_X program (Thompson et al., 1997). The phylogenetic trees were constructed using the neighbour-joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) methods implemented by the PHYLOWIN program (Galtier et al., 1996) and 500 bootstrap replicates. All methods (NJ, ML and MP) gave similar profiles. A phylogenetic tree was drawn using TreeView (Page, 1996) (Fig. 1). The KBV sequences from the French isolate described in this paper were submitted to the GenBank database under accession no. KC130158 (primers KBV1 and KBV2 described by Stoltz et al., 1995) and no. KC130157 (primers KBV-F2952 and KBV-R3610 described in this paper).

Table 1
Origin of the ABPV, IAPV and KBV isolates used for the phylogenetic analysis: sequence label, country of origin, reference and Genbank accession number.

Isolate	Country	Reference	GenBank accession no.
ABPV	United Kingdom	Govan et al. (2000)	AF150629
FRA5	France	Tentcheva et al. (2004)	AY669853
IAPV	Israel	Maori et al. (2007)	NC_009025
FRA1	France	Blanchard et al. (2008)	EU604007
AUS1	Australia	Hung et al. (2000)	AF034541
FRA2	France	Tentcheva et al. (2004)	AY669845
FRA3	France	Tentcheva et al. (2004)	AY669846
JOR	Jordan	Al-Abbadi et al. (2010)	FJ225117
RUS	Russia	Hung (1999)	AF197905
KOR	South Korean	Ju et al. (2008)	EU770972
KBV	United States (Pen)	de Miranda et al. (2004)	AY275710
DAN	Denmark	Nielsen et al. (2008)	EF570891
GER	Germany	Siede and Büchler (2004)	AY787143
CAbee2	United States (Cal)	Hung et al. (2000)	AF135852
CAbee7	United States (Cal)	Hung et al. (2000)	AF135857
CAbee13	United States (Cal)	Hung et al. (2000)	AF052566
MDbee	United States (Mar)	Hung and Shimanuki (1999)	AF027125
MEbee	United States (Mai)	Hung et al. (2000)	AF035359
CANbee	Canada	Hung et al. (2000)	AF034542
AUS2	Australia	Palacios et al. (2008)	EU436457
SPA	Spain	Esperon et al. (2004)	AY821562
FRA4	France	This report	KC130158
NWZ	New Zealand	Siede and Büchler (2004)	KC513761

The phylogenetic analysis demonstrated that the French isolate collected in 2012 belongs to the KBV genotype, while isolates from France (Tentcheva et al., 2004), South Korean (Ju et al., 2008), Russia (Hung, 1999) and Jordan (Al-Abbadi et al., 2010), originally identified as KBV belong to the IAPV genotype. In addition, this phylogenetic analysis confirms that the Australian KBV-like sequence (GenBank no. AF034541) belongs to the IAPV genotype, while a recent study in Italy (Cersini et al., 2013) reports a misclassified KBV isolate showing 97% of identity with this Australian sequence. These results are in agreement with those described by de Miranda et al. (2010), highlighting again the initial misclassification of IAPV and KBV sequences. de Miranda et al. (2010) also suggest that KBV primers described by Stoltz et al. (1995) should now be discontinued for diagnostics purpose, except for sequencing and phylogenetic analysis to compare with sequences available in databases.

In this study, KBV infection in bees was demonstrated for the first time in France. It has been shown that the KBV positive sample was ABPV and IAPV negative, while IAPV positive samples were KBV negative. These results confirm that the KBV primers described in this study can be used for KBV specific detection. This KBV PCR assay was also validated according to the AFNOR XP U47-600 standard. In addition, the multiplex PCRs described by Carletto et al. (2010) were tested on almost 400 honeybee samples collected in France

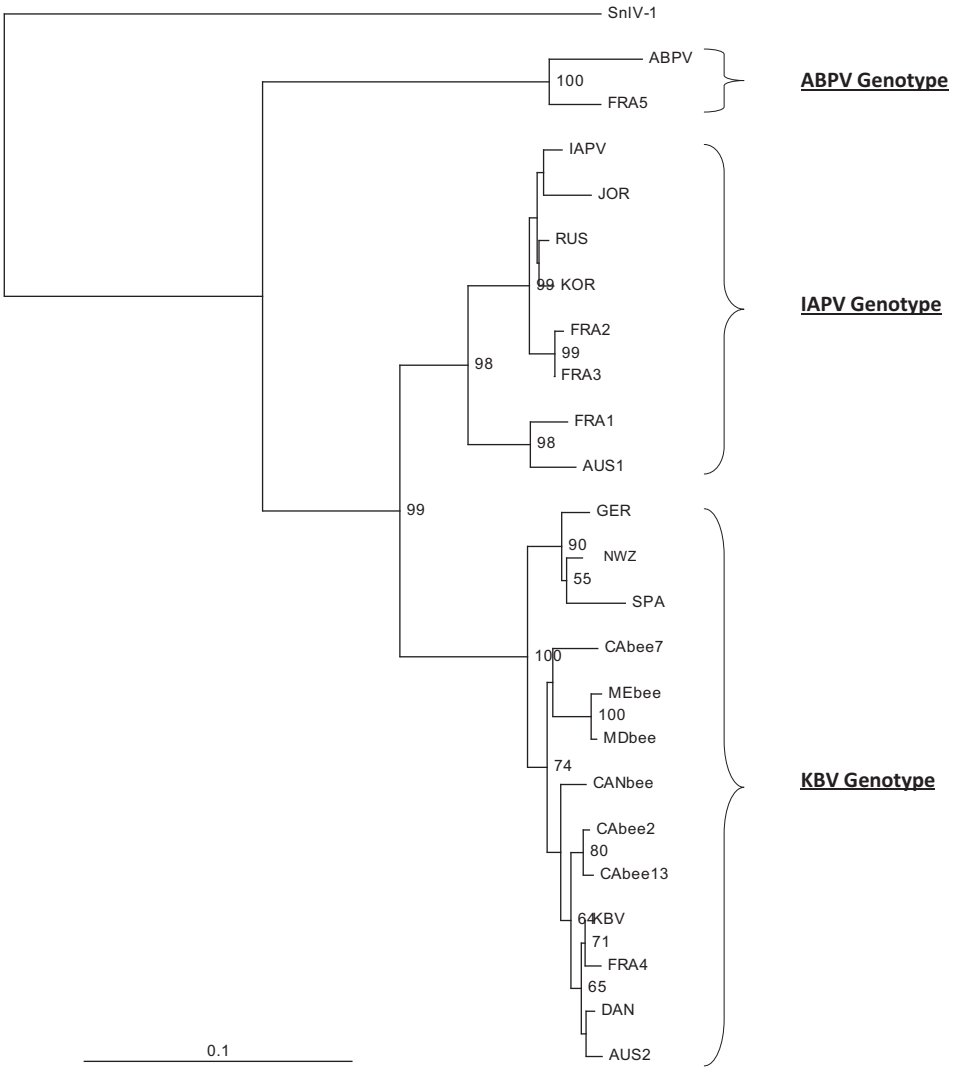

Fig. 1. Neighbour-joining phylogenetic tree of KBV sequences, based on the 374 bp polymerase region (nucleotides 5406–5819 according to reference sequence AY275710) compared to IAPV and ABPV sequences. The number of each node represents the bootstrap value as the result of 500 replicates. Bootstrap values <50% were omitted. The scale corresponds to the number of substitution per site. *Solenopsis invicta* virus-1 (SnIV-1) was used as outgroup.

between 2009 and 2010, and no sample was found positive for KBV. These multiplex PCRs used two KBV primer pairs, thus ensuring more reliable detection in the event of genetic variability, the first pair described in this study and the second pair described by Shen et al. (2005).

In this study, the KBV positive sample was negative for other honeybee viruses (ABPV, IAPV, CBPV, DWV, SBV and BQCV) and other pathogens such as *V. destructor*, *N. apis* and *N. ceranae* while positive samples for one or several bee pathogens were KBV negative. KBV has already been detected in neighbouring countries of France (Spain, England, Germany and Italy). This virus was also reported in France (Tentcheva et al., 2004), but using non specific primers which actually detected IAPV rather than KBV, contrary to the present study, as supported by phylogenetic analysis. Specific primers are necessary for accurate molecular diagnostic of viral infections in bees. On the other hand, intra-specific recombination such as found in the ABPV–KBV–IAPV complex (Maori et al., 2007; Palacios et al., 2008) could also contribute to the misclassification of these closely related viruses. Finally, further investigations are necessary to understand the presence of KBV in an apparently healthy apiary in France in April 2012 and the lower occurrence of KBV in France.

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