Apidologie 38 (2007) 181–190
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DOI: 10.1051/apido:2006072

Original article

First detection of Kashmir bee virus in the UK using real-time PCR*

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Received 24 May 2006 – Revised 2 August 2006 – Accepted 23 August 2006

Abstract – *Kashmir bee virus* (KBV) often persists in bees as a covert infection with no apparent symptoms. The virus can switch to become an overt lethal infection, especially in the presence of *Varroa* mites. Although the virus is distributed worldwide, it was thought to be absent from the UK. A real-time PCR assay was developed for specific detection of KBV. No cross-reaction was observed with other bee viruses. KBV was successfully amplified from different life stages of honey bees and from a wasp and bumble bee. Using the real-time PCR assay, a survey of hives was conducted in England and Wales to investigate the presence and geographical distribution of the virus. KBV was detected within three colonies at two locations. The virus titre in the positive samples was quantified and found to contain similar levels to other bees with covert KBV infection. We conclude that KBV is present in the UK and cannot now be considered an exotic disease. The discovery of KBV in the UK has major significance for import policies.

Kashmir bee virus / *Apis mellifera* / real-time PCR / detection / survey

1. INTRODUCTION

Honeybee viruses have had a high profile in recent years due to their association with *Varroa destructor* Anderson and Trueman in colony collapses (Allen and Ball, 1996; Ball, 1997; Ball and Bailey, 1997; Nordström et al., 1999; Martin, 2001; Bakonyi et al., 2002; Sumpter and Martin, 2004) and in risk analysis for third country imports under the General Agreement on Tariffs and Trade – World Trade Organisation (GATT-WTO) sanitary and phytosanitary (SPS) agreements (Brown, 1999; Wehling, 2000). Despite this, many of the bee viruses have only recently been sequenced (Ghosh et al., 1999; Govan et al., 2000; Leat et al., 2000), and the nomenclature established (Van Regenmortel et al., 2000; Mayo, 2002), but crucially, only several surveys of bee virus incidence have been completed (Bruce et al., 1995; Allen and Ball, 1996; Bakonyi et al., 2002; Tentcheva et al., 2004; Berényi et al., 2006).

Kashmir bee virus (KBV) was first confirmed in *Apis cerana* Fabricius (Bailey and Woods, 1977), and analysis of the genome sequence shows that it is a cricket paralysis-like virus (family Dicistroviridae: genus Cripavirus) (de Miranda et al., 2004; Lanzi et al., 2006). Like many other bee viruses, KBV can persist within bees as 'covert' infections, which may be activated in various ways to an overt and lethal infection (Dall, 1985; Ball, 1997). There is some debate as to the extent of the pathological nature of this virus; opinions vary from it being relatively innocuous to highly pathogenic (Hornitzky, 1987; Anderson and Gibbs, 1988; Anderson, 1991; Ball, 1997). KBV is found in many parts of the world, including North America (Bruce et al., 1995; Hung et al., 1996b),

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Table I. Sequence of the TaqMan primers and probes designed for detection and quantitation of *KBV* in honey bees. F: forward primer; R: reverse primer; T: probe. Probes consist of oligonucleotides with a 5'-reporter dye and a 3'-quencher dye (TAMRA: tetra-methylcarboxyrhodamine).

Primer and probe	Target	Sequence (5'-3')
AF263725-kbv-83F	KBV	ACCAGGAAGTATTCCCATGGTAAG
AF263725-kbv-161R	KBV	TGGAGCTATGGTTCCGTTCAG
AF263725-kbv-109T[a]	KBV	CCGCAGATAACTTAGGACCAGATCAATCACA
AJ307465-955F	Bee IPC	TGTTTTCCCTGGCCGAAAG
AJ307465-1016R	Bee IPC	CCCCAATCCCTAGCACG AA
AJ307465-975T[b]	Bee IPC	CCCGGGTAACCCGCTGAACCTC

[a] Reporter dye is FAM: 6-carboxy-fluorescein.
[b] Reporter dye is JOE™.

Spain (Allen and Ball, 1995), Germany (Siede and Büchler, 2003), France (Gauthier et al., 2003), India (Allen and Ball, 1996), Fiji (Allen and Ball, 1996), Australia and New Zealand (Hornitzky, 1981; Anderson, 1983; Hornitzky, 1987; Anderson, 1988; Todd and Ball, 2003). Distinct sources of KBV have been isolated in different parts of the world, distinguishable from one another by comparison of the coat protein (Allen and Ball, 1995).

The aim of this work was to carry out a survey of hives in England and Wales to ascertain the status of KBV. Real-time PCR primers and a probe were designed for the specific detection of KBV and to amplify the 18S rRNA gene from the honey bee host for use as an internal positive control. An automated extraction method, based on the use of silica coated paramagnetic beads was developed to offer more rapid screening of large numbers of bees.

2. MATERIALS AND METHODS

2.1. Viral samples

Purified KBV isolates were obtained from different locations and insect species. Isolate KBV 14 (from honey bee in Australia, exact location unknown) 16 (from bumble bee in Auckland, New Zealand) and KBV NZ (from honey bee in Auckland, New Zealand). Other purified virus preparations and virus infected bees and pupae were supplied by Rothamsted Research (UK), USDA (America) and HDLGN (Germany). Infected bee samples were transported at ambient temperatures in 70% ethanol, and stored at –20 ˚C before analysis.

2.2. Real-time PCR primer and TaqMan® probe design

Sequence data for coat protein genes from KBV and the closely related Acute paralysis virus (ABPV) were downloaded from the EMBL database to compare inter and intra viral sequence variability. Multiple sequence alignments were carried out using the CLUSTAL V algorithm in the package MegAlign (DNASTAR inc., Madison, USA). Real-time PCR primers and a probe were designed (Primer Express™ software, Applied Biosystems, Branchburg, USA) to a region of sequence (using accession AF263725) conserved in KBV but distinct from ABPV. The assay was tested for cross-reaction against ABPV and the other bee viruses Black queen cell virus (BQCV), Sacbrood virus (SBV), Cloudy wing virus (CWV), Slow paralysis virus (SPV), Deformed wing virus (DWV), Chronic paralysis virus (CPV) and *Apis* iridescent virus (AIV).

Real-time PCR primers and a probe were designed to the bee 18S rRNA gene of *Apis mellifera* (AJ307465) to serve as an internal positive control (IPC) for assessing nucleic acid extraction efficiency. The 5' terminal reporter dyes for the probes were 6-carboxyflurorescin (FAM) and JOE™ and the 3' quencher was tetra-methylcarboxyrhodamine (TAMRA) (Tab. I).

2.3. Survey of colonies for KBV in England and Wales

Adult honey bees were collected from colonies throughout England and Wales; samples were collected by Appointed Bee Inspectors and individual

beekeepers. Samples were taken from both healthy and unhealthy hives. All bee samples were placed directly in 40 mL polypropylene universal bottles containing 25 mL of 70% ethanol and stored at 4 °C prior to testing.

2.4. Tissue homogenisation

The non survey samples were tested individually whilst the survey samples were tested by bulking 5 bees together for each sample, such that larger number of bees could be tested from each hive. *Non-survey samples*: individual bees/wasps were placed inside 2 mL screw cap micro-centrifuge tubes with 3 tungsten carbide beads (3 mm) (Qiagen Ltd, Crawley, UK) and 1 mL of lysis buffer (ThermoLabsystems, Vantaa, Finland); the tubes were mounted onto a mixer mill (Qiagen) and shaken at an amplitude of 30/s for 3 minutes. The tubes were centrifuged at 6000 *g* (room temperature) for 3 min to remove debris. *UK survey samples*: for the survey samples, two replicates each containing 5 bees, were processed per survey sample. Excess ethanol was removed from all bees by blotting on paper towel. Each replicate of 5 bees were placed in a 100 mm × 150 mm filter grinding bag (Bioreba, Reinach, Germany) with 5 mL of lysis buffer (Thermo Labsystems) and ground manually with a Homex grinder (Bioreba). The lysate was decanted into a 5 mL screw cap tube. Tubes were spun at 6000 *g* for 1 min to pellet debris.

2.5. RNA extraction from Bees

RNA was extracted from the cleared lysate using a silica-based total RNA extraction kit (Thermo Labsystems) in conjunction with a magnetic particle processor (Kingfisher ML, Thermo Labsystems). The manufacturer's program 'Total_RNA_ML_1' was used. Briefly, Kingfisher tubes were prepared as follows – tube 1: 1000 μL of cleared lysate and 50 μL of MagnaSil beads (Promega); tube 2: 600 μL of lysis buffer b (Promega); tubes 3 and 4: 1 mL of 70% ethanol; tube 5: 200 μL of molecular grade sterile water (BDH, Lutterworth, UK). The resulting RNA eluted into tube 5 was transferred to a fresh 1.5 mL tube and stored at –20 °C prior to use.

2.6. Real-time PCR assays

All assays were run as simplex assays. Reactions were set up in either 96 or 384 well reaction plates using PCR core reagent kits (Applied Biosystems), following the protocols supplied, but with the addition of 0.5 units of M-MLV reverse transcriptase (Promega) per reaction. For each reaction, 1 μL of total RNA was added, giving a final volume of 25 μL. Plates were then cycled at generic system conditions (48 °C/30 min, 95 °C/10 min and 40 cycles of 60 °C/1 min, 95 °C/15 s) within the 7900 HT Sequence Detection System (Applied Biosystems), using real-time data collection.

2.7. Cloning and sequencing PCR products

Real-time PCR products were directly ligated into plasmid pGEM®-T Easy Vector (Promega), and transformed into *E. coli* JM109 High Efficiency competent cells (Promega) following the manufacturer's protocols. White bacterial colonies containing plasmids with inserts were selected and plasmid DNA was purified using the Wizard®Plus SV miniprep DNA purification system (Promega). Purified plasmid concentrations were adjusted to 20 ng μL^{-1} for sequencing. Sequencing was carried out by the DNAseq sequencing service, University of Dundee, Scotland.

2.8. Quantification of KBV virus load in bees

The KBV and bee IPC real-time PCR assays were used to quantify KBV virus load in positive UK samples using a relative standard curve method (User Bulletin 2, Applied Biosystems). For this method, standard dilutions were prepared from RNA extracted from a purified KBV preparation (KBV 14) and from bees collected from the CSL apiary. Each standard was diluted through five levels of a two-fold dilution series. The standard dilutions were tested alongside the UK KBV positive RNA samples, and for comparison, alongside RNA extracted from Australian bees known to be naturally infected with high levels of KBV or covertly infected (Dennis Anderson personal communication). Standard curves were constructed for KBV and bee IPC by plotting the log of each standard dilution verses the TaqMan® C_t value (threshold cycle at which the first PCR products were detected).

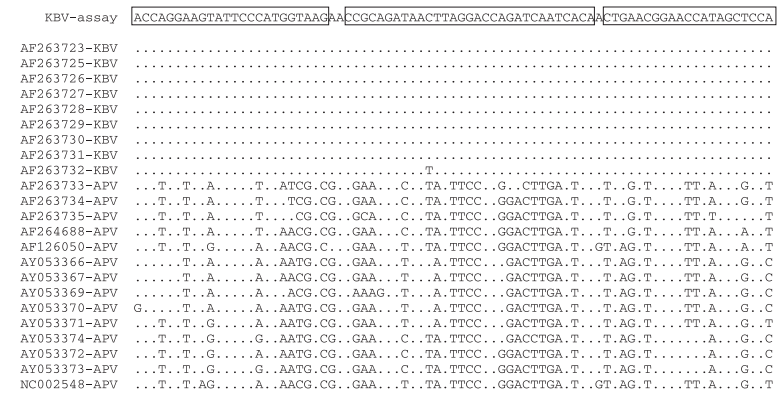

Figure 1. Multiple alignment of all-available KBV sequences alongside ABPV sequences (for ABPV, if more than one sequence is available with 100% identity in this region, only one of each sequence 'type' is shown), indicating the positions of the primers and probes designed (in boxes). Only polymorphic nucleotides are shown, (.) indicates an identical nucleotide at that position.

The quantity of KBV virus in the UK and Australian samples were extrapolated from the standard curve. The virus concentrations were normalised by dividing the inverse log of virus quantity by the inverse log of the bee IPC quantity.

3. RESULTS

3.1. Real-time primer and probe design

Although KBV and ABPV are closely related it was possible to discriminate between the two viruses by designing TaqMan® primers and a probe to conserved region of KBV sequence but where the ABPV sequence was variable (Fig. 1, Tab. I). The KBV assay gave a positive PCR result when tested with 3 purified virus preparations, KBV14, KBV16 and KBVNZ. No cross-reaction was observed (no amplification after 40 PCR cycles) when RNA was extracted from pure virus or bees infected with the following viruses ABPV, BQCV, SBV, CWV, SPV, DWV, CPV and AIV. To confirm the presence of virus in the pure virus preparations electron microscopy was performed, and in each case virus particles of the correct size were observed (data not shown). For the viruses where sequence was available (BQCV, SBV, DWV and AIV) the presence of virus was confirmed by RT-PCR/PCR (data not shown). For Cloudy wing virus (CWV) electron microscopy confirmed the presence of virus particles of the correct size, whilst the KBV assay did not detect viral RNA, even though the available sequence of CWV is identical to KBV. Although AIV is a DNA virus it could still be detected in the RNA extractions from infected bees using PCR (without an RT step), indicating that sufficient DNA was co-purified in the extraction procedure to enable effective detection.

KBV was successfully detected in different life stages of *Apis mellifera* L. including Australian worker honey bees, worker pupae and drone pupae. Some of the worker honey bees from America also tested positive for the virus, along with one Australian wasp (*Vespula germanica*). This further indicates that the assay developed is able to detect different sources of KBV in *A. mellifera* as well as KBV in different insect species. Detection in other insect species is further supported by the successful amplification of KBV sequence from the virus preparation KBV16, obtained from a bumblebee. The German bees all tested negative for KBV, however, the internal control results show that adequate levels of total RNA had been extracted (Tab. II).

3.2. Survey of honeybee colonies for KBV

RNA extracted from 5 bees in each survey sample was tested with the real-time PCR

Table II. Results from testing known infected samples and samples suspected of being KBV-infected, including the number of positive samples and the average C_t values and standard deviations for the KBV assay in positive samples and the internal positive control assay in all samples extracted. The instrument records a C_t value of 40 for the negative samples (-).

Source	Sample type	Number of positive individuals tested	Average C_t for KBV positive samples	Average C_t for 18S assay for all samples
Australia	Wasp pupa	1/2	24.36	23.52 ± 0.18
Australia	Honey bee worker pupa	6/6	29.09 ± 3.28	14.64 ± 0.13
Australia	Honey bee drone pupa	3/4	36.18 ± 1.66	14.48 ± 0.23
Australia	Honey bee worker	3/10	35.36 ± 6.54	16.48 ± 1.20
Australia	Honey bee worker	6/15	36.50 ± 1.63	23.24 ± 3.49
USA	Honey bee worker	2/10	31.85 ± 1.45	16.39 ± 1.55
Germany	Honey bee worker	0/10	-	17.59 ± 5.12
Germany	Honey bee worker	0/10	-	17.38 ± 0.75

assays for the bee 18S rRNA (IPC). Using the automated extraction method, RNA was successfully recovered from all bee samples. A similar extraction efficiency was observed for all samples indicating that the method was reproducible. The average real-time PCR C_t (denoted by the point at which amplification is first observed above the base threshold) value for two replicates from 458 survey samples was 10.39 ± 1.52.

Following testing using the KBV real-time PCR assay, of the 458 samples tested, three tested positive for the presence of KBV. Two of the positive samples were detected in different hives from the same apiary. Details of these colonies are shown in Figure 2. One of the sampled colonies was infected with European foulbrood (a statutory notifiable disease) and was destroyed as part of the national disease control strategy. PCR products amplified during the real-time PCR testing were cloned and sequenced. The sequence of the cloned PCR product sequence was confirmed to be KBV following a blast search on the NCBI database which matched other KBV sequences (Accessions AY275710, AF263723–AF263732, AY452696).

3.3. Quantification of virus load in UK bee samples

The quantity of KBV in the positive UK samples were compared to Australian bees that were known to be naturally infected with KBV at low levels (non-symptomatic) or high levels (showing clinical symptoms). A standard curve method was used to quantify virus load in the bees rather than the ΔC_t method as described in Chen et al. (2005). When the relative efficiencies of the bee IPC and KBV were plotted as ΔC_t [$C_{t_{(\mathrm{KBV})}} - C_{t_{(\mathrm{IPC})}}$], versus the log of the RNA dilution, the ΔC_t had a value greater than 0.1 (the slope was 0.9513), indicating that the amplification efficiencies of KBV and bee IPC were not equal. In this instance, the ΔC_t method was not suitable for quantification. In addition, as the starting level of virus and bee IPC RNA in the standard dilutions was unknown, relative rather than absolute quantification was used.

A standard curve was constructed by plotting C_t values of bee and KBV standard RNA dilutions against the log of the RNA dilution. The quantity of KBV and bee IPC RNA from each bee was extrapolated from the standard curve. The normalised quantity of KBV in each bee was determined by dividing the amount of KBV RNA by the amount of bee IPC RNA. The results show that the virus levels in the infected bees from the UK are similar to those in bees with covert levels of infection from Australia. In comparison, other bees (also from Australia) believed to be highly infected, contained around 400 times more virus than the bees at the UK Manchester site and 100 times more virus than covertly infected Australian bees and bees from the UK Hull site (Fig. 3).

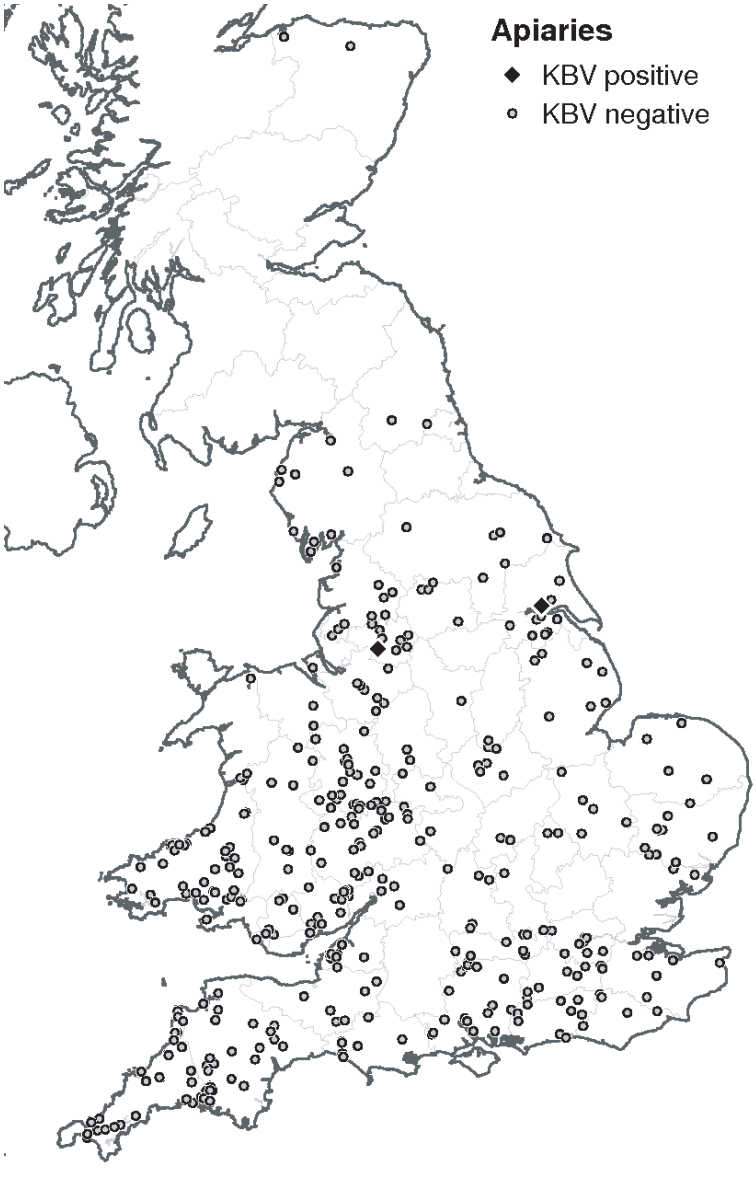

Figure 2. A Map showing the locations of the 458 bee colonies sampled in the UK 2004 KBV survey including the two colonies that tested positive for KBV (indicated by black diamonds).

4. DISCUSSION

This paper reports the first finding of KBV in the UK and the first large-scale survey for bee viruses in Europe using TaqMan technology. This study demonstrates the practical application of this technology as a support tool for surveys and contingency management, and to provide robust surveillance data on the presence or otherwise of KBV and other honey bee viruses.

The incorporation of an internal control assay to detect the bee 18S rRNA gene allowed the assessment of extraction efficiency and allowed interpretation of false negative results.

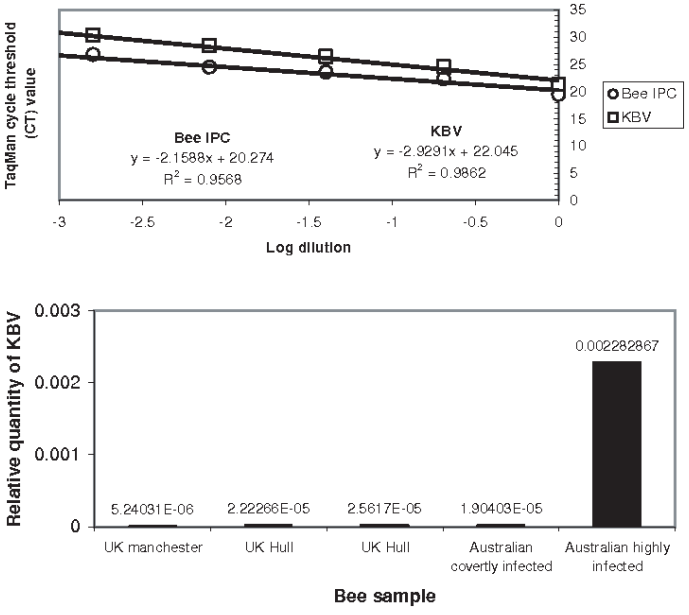

Figure 3. Relative quantification of KBV in bee samples. (A) TaqMan Standard curves for KBV virus and bee IPC. (B) Relative comparison of KBV levels in UK bees in relation to bees known to be naturally infected with high and covert levels of KBV infection.

Also, the real-time PCR assays allowed the quantitative assessment of virus levels over several orders of magnitude. This proved to be valuable for detection of low-titre infections within the bees tested. In this study, quantification of KBV levels in the UK bees showed they contained similar levels to Australian bees having covert infections. The bee colonies from the UK sites showed no obvious signs of virus infection at the time of collection suggesting the virus was covert or latent in these apiaries. It is known that the viruses can exist in individual bees or entire colonies for long periods without causing noticeable clinical symptoms (Dall, 1985; Anderson and Gibbs, 1988; Hung et al., 1996a, b). Studies by Anderson and Gibbs (1988) showed that covert virus infections were common in pupae with KBV. The status and sites where the virus may remain in the bee in this state are unknown, however, it has been suggested the lining of the gut may be the site of 'unapparent' or covert infection (Anderson and Gibbs, 1989). Anderson and Gibbs (1988) have stated that these infections do possess a latent capacity to become activated and develop into acute infections.

Some studies have suggested that viruses such as KBV and ABPV may exist as natural infections in a broader range of genera other than *Apis*. For example KBV has been detected in the wasp *Vespula germanica* (Anderson, 1991) and ABPV in *Bombus* species (Bailey and Gibbs, 1964). Our study provides further support for this, as KBV was detected in the same species of wasp and one of the KBV serotypes tested was extracted from a bumblebee from New Zealand. In terms of trade and regulation of honeybee viruses between countries, if these viruses exist in different insect hosts, it makes such controls and restrictions difficult to impose or justify. In view of this, the full host-range of honeybee viruses warrants further study.

At the time of sampling, all three KBV positive UK colonies were in good condition, with healthy numbers of adult bees and brood, however, the colonies were infested with varroa and one of the colonies was found to have European foulbrood (EFB). The association of

KBV with other honeybee pathogens including EFB has been reported before in Australia (Hornitzky, 1981).

We conclude that KBV is present in the UK and cannot now be considered an exotic disease. There is growing consensus that KBV may not be the threat it was initially perceived to be and may be no more of a threat than the other extant bee viruses present in the UK. The discovery of KBV in the UK does have major significance for import policies, demonstrating the need for accurate surveillance data on pathogen incidence to inform the formal import risk analysis and policy decision-making process under the Office International des Epizooties (OIE) and WTO agreements.

ACKNOWLEDGEMENTS

This work was supported by the Department for Environment, Food and Rural Affairs (England) and Department for Environment, Planning and Countryside (Wales) as part of the Bee Health Programme run for both countries by the Central Science Laboratory. The authors would like to thanks Dr. Denis Anderson (CSIRO, Australia), Dr. Judy Chen (USDA, Beltsville), Dr. Reinhold Siede (HDLGN, Kirchhain) and Dr. Brenda Ball (Rothamsted Research Institute) for their kind donations of viruses and virus-infected bees, and all the beekeepers and Bee Inspectors that took part in the survey work. Thanks also to Moray Taylor for supplying the KBV survey map and P.C. would like to acknowledge the Thailand Research Fund.

Première identification du virus du Cachemire de l'abeille en Grande-Bretagne par PCR en temps réel.

Apis mellifera **/ virus du cachemire / identification / PCR en temps réel / enquête**

Zusammenfassung – Der erste Fall von Kaschmir-Bienenvirus in Grossbritannien entdeckt mittels Real-Time-quantitativer PCR. Wie viele andere Bienenviren ist auch das Kaschmir-Bienenvirus (KBV) in vielen Bienen als latente Infektion ohne offensichtliche Symptome präsent. In der Anwesenheit von Varroamilben kann der Virusbefall sich jedoch zu einer lethalen Infektion entwickeln. Das KBV ist zwar weltweit verbreitet, für Grossbritannien wurde jedoch bislang kein Vorkommen gemeldet.

Wir entwickelten ein Protokoll zur spezifischen Detektion von KBV mittels Real-Time-quantitativer PCR. Dieses zeigte keine Kreuzreaktionen mit RNA anderer Bienenviren, wie ABPV, BQCV, SBV, CWV, SPV, DWV, CPV und AIV, und ermöglichte die Ampflifikation von PCR-Produkten aus virenbefallenen Bienenproben der verschiedenen Stadien des Lebenszyklus, ebenso wie von KBV-infizierten Bienen aus verschiedenen geographischen Regionen. PCR-Fragmente konnten auch aus RNA-Proben einer Wespe (*Vespula germanica*) aus Australien und aus einer aus Hummeln aufgereinigten KBV16 Virusprobe amplifiziert werden. Alle PCR-Produkte wurden kloniert und mittels Sequenzierung als KBV-RNA identifiziert.
Mittels dieses Protokolls einer Real-Time-quantitativer PCR analysierten wir Proben aus 458 Völkern aus ganz England und Wales, um die eventuelle Anwesenheit und die geographische Verbreitung des KBV-Virus zu erfassen. Diese Übersichtsstudie zeigte die Präsenz des Virus in drei Völkern von zwei Ständen an. Mittels Real-Time-quantitativer PCR konnten wir zeigen, dass der Virentiter in diesen positiven Proben ähnliche KBV-Werte aufwies wie in Bienenproben aus Australien, die einen klaren Befall gezeigt hatten. Wir schliessen daraus, dass das KBV in Grossbritannien vorkommt, und dass es jetzt nicht länger als eine exotische Krankheit betrachtet werden kann. Die Klärung der Auswirkung des KBV-Befalls auf Völker in Grossbritannien erfordert weitere Untersuchungen, aber es scheint nicht die grosse Bedrohung zu sein, für die es bislang gehalten wurde, und es kann durchaus auch sein, dass das KBV keine grössere Bedrohung darstellt als andere, bereits in Grossbritannien vorkommende Viren. Die Entdeckung des KBV in Grossbritannien ist jedoch von Bedeutung in Hinsicht auf Importregelungen und belegt die Notwendigkeit von genauen Übersichtsdaten über das Vorkommen von pathogenen Agentien für die Erstellung formaler Importrisikoanalysen und für Entscheidungsprozesse innerhalb der Vereinbarungen des Internationalen Büros für Epizootien (OIE) und der WTO.

Kaschmir Bienenvirus / ***Apis mellifera*** **/ Real-Time-quantitativer PCR / Übersichtsdaten**

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