SINGLE ASSAY DETECTION OF ACUTE BEE PARALYSIS VIRUS, KASHMIR BEE VIRUS AND ISRAELI ACUTE PARALYSIS VIRUS

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Received 12 February 2012, accepted 11 May 2012

S u m m a r y

A new RT-PCR primer pair designed to identify Acute Bee Paralysis Virus (ABPV), Kashmir Bee Virus (KBV) or Israeli Acute Bee Paralysis Virus (IAPV) of honey bees (*Apis mellifera* L.) in a single assay is described. These primers are used to screen samples for ABPV, KBV, or IAPV in a single RT-PCR reaction saving time and money. The primers are located in the predicted overlapping gene (*pog*/ORFX) which is highly conserved across ABPV, KBV, IAPV and other dicistroviruses of social insects. This study has also identified the first case of IAPV in Denmark.

Keywords: honey bee virus, IAPV, KBV, ABPV, AKI primers.

INTRODUCTION

Acute Bee Paralysis Virus (ABPV), Kashmir Bee Virus (KBV) and Israeli Acute Bee Paralysis Virus (IAPV) are closely related viruses affecting the western honey bee (*Apis mellifera* L.). Acute Bee Paralysis Virus, KBV and IAPV are non-enveloped, positive stranded RNA viruses that belong to the Dicistroviridae family of viruses. The quasi-species status in the ABPV complex of the three viruses is not firmly established, as discussed by De Miranda et al. (2010). Colony collapse disorder (CCD) is the abrupt disappearance of adult bees resulting in collapse of the hive (Vanengelsdorp et al., 2008).

Israeli Acute Bee Paralysis Virus has been shown to be related to CCD (Cox-Foster et al., 2007). For this reason, it is vital to have fast and efficient methods of monitoring and detecting the related IAPV, ABPV and KBV viruses.

Numerous primers have been designed over the years to detect these viruses from bee samples (Tentcheva et al., 2004, Cox-Foster et al., 2007, Grabensteiner et al., 2007, Maori et al., 2007).

Bee disease diagnostic labs receive bee samples to be tested for viruses using different primer pairs in usually separate reactions. In the case of ABPV, KBV and IAPV, three different PCR reactions have to be set up for each sample.

The three viruses, ABPV, KBV and IAPV are closely related and show very high sequence similarity (De Miranda et al., 2010).

The aim of this experiment included designing a primer pair in a highly conserved region allowing the detection of ABPV, KBV, and IAPV in a single assay. This is an efficient way to reduce cost and time for primary screening of multiple samples for presence or absence of these three viruses. The positive samples can then be subjected to amplicon sequencing or specific PCR to determine the specific virus. The first case of IAPV in Denmark is also described in this study.

MATERIALS AND METHODS

Bee Material

Live bee samples were collected from experimental hives located in Flakkebjerg, (South Zealand, Denmark), some with a high load of varroa mites. Live samples sent by Danish beekeepers for diagnosis were also used in this study. Samples were

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also received from other countries (bee or RNA material) for the purpose of testing variant strains. Samples were stored at -80 °C.

Primer Design

The following complete genomes of IAPV ([Genbank: EU436456.1] (Australia), [Genbank: EU436455.1] (Israel), [Genbank: EU436423.1] (USA), [Genbank: NC_009025.1] (Israel)), ABPV ([Genbank: AF486073.2] (Poland), [Genbank: AF486072.2] (Hungary), [Genbank: HB342184.1], [Genbank: HB342179.1], [Genbank: NC_002548.1], [Genbank: AF150629.1]) and KBV ([Genbank: NC_004807.1] (USA), [Genbank: HB_342185.1], [Genbank: AY275710.1] (USA)) were obtained from the NCBI database. These genomes were aligned in ClustalW (Thompson et al., 2002) and a consensus sequence was derived. The consensus sequence was used in the Primer3Plus website (Untergasser et al., 2007) to design the primers. The primer sequences were compared to all currently known sequences of the three viruses thus covering several regions such as USA, Australia, Poland, Hungary and Israel. The two primers are F: CTTTCATGATGTGGAAACTCC and R: AAACTGAATAATACTGTGCGTA. These primers are referred to as AKI primers in this paper. The position of the primers on the IAPV [Genbank: NC_009025], ABPV [Genbank: NC_002548] and KBV [Genbank: NC_004807] genomes are shown in Fig. 1.

RT-PCR Analysis

Bee samples (fresh or frozen) were placed in liquid nitrogen and crushed using a micropestle in an eppendorf tube. RNA was extracted using Nucleospin RNA II Kit (Macherey-Nagel). The reverse transcriptase – polymerase chain reaction (RT-PCR) was carried out using OneStep RT-PCR kit (Qiagen) using the following conditions for AKI and IAPV primers, initial 30 min incubation at 50ºC, followed by 15 min at 95ºC and 35 cycles of 30 s at 94ºC, 30 s at 59ºC, and 20 s at 72ºC followed by 4 min at 72ºC. The RT-PCR conditions for ABPV, Deformed Wing Virus (DWV), KBV and Sacbrood Virus (SBV) were as follows, initial 30 min incubation at 50ºC, followed by 15 min at 95ºC and 35 cycles of 30 s at 94ºC, 45 s at 56ºC, and 1 min at 72ºC followed by 7 min at 72ºC. The final concentration of primers in a 25 µl reaction was 0.4 µM for AKI primers and 0.6 µM for others. The Qiagen OneStep RT-PCR kit is provided with a 'Q-Solution' which aids amplification of GC rich regions. The Q-solution yielded fuzzy bands with AKI primers, so it was not used. All other reagents were used in concentrations prescribed by the kit. Thermal cycling was carried out on a 2720 Thermal Cycler (Applied Biosystems). The PCR products were separated by 2% agarose gel electrophoresis on RunOne Electrophoresis system (EmbiTech). Polymerase chain reactions (PCR) were performed from 87 samples which included adult bees, larvae and pupae. Some foreign samples were provided as extracted RNA. This includes bee samples previously tested positive for KBV or ABPV using specific primers. Purified IAPV-integrated plasmid clones provided by Eyal Maori were used as the positive control for IAPV as no IAPV positive bee samples were available. The IAPV samples identified in this study were available only towards the end the experiment and therefore could not be used as positives. Verification was performed using the primers for ABPV and KBV published by Tentcheva et al. (2004) and for IAPV published by Maori et al. (2009). Samples positive for other common viruses such as DWV (Deformed Wing Virus) (Tentcheva et al., 2004) and SBV (Sacbrood Virus) (Grabensteiner et al., 2001) were also tested to eliminate false-positives. The list of primers used in this study has been summarised in Table 1. Samples infected with multiple viral infections were also tested for specificity of the primers. Samples originating outside Denmark including Benin, France, Germany, Hungary, Italy, South Africa, Switzerland and Turkey were tested using these primers.

Table 1.

List of Primers used in this study. ABPV, KBV, DWV, IAPV, SBV and AKI Primers. *Annealing Temperature (AT) used and use of Q-solution (Qiagen Kit) is indicated

Primers		Sequence	AT* (°C)	Q-solution
ABPV[1]	F	TGAGAACACCTGTAATGTGG	56	+
	R	ACCAGAGGGTTGACTGTGTG		
KBV[1]	F	GATGAACGTCGACCTATTGA	56	+
	R	TGTGGGTTGGCTATGAGTCA		
IAPV[2]	F	AGACACCAATCACGGACCTCAC	59	+
	R	GAGATTGTTTGAGAGGGGTGG		
DWV[1]	F	TTTGCAAGATGCTGTATGTGG	56	+
	R	GTCGTGCAGCTCGATAGGAT		
SBV[3]	F	ACCACCCGATTCCTCAGTAG	56	+
	R	CCTTGGAACTCTGCTGTGTA		
AKI	F	CTTTCATGATGTGGAAACTCC	59	-
	R	AAACTGAATAATACTGTGCGTA		

[1](Tentcheva et al., 2004), [2](Maori et al., 2009), [3](Grabensteiner et al., 2001).

Sequence Analysis

Forty-eight PCR products were sequenced to determine the base composition and to verify the virus. The sequence analysis workflow was managed using Geneious Pro 5.1.3 (Drummond et al., 2011). The forward and reverse sequence obtained for each PCR product were aligned and merged into a single sequence. The primer regions from these sequences were discarded and the resulting fragments were batch blasted against the NCBI nucleotide database using the BLAST engine (Altschul et al., 1990).

In case of ambiguous results, the sequences were aligned and corrected by eye against those complete viral genomes indicated previously.

RESULTS

A homology comparison between these three viruses showed that the percentage similarity between ABPV-IAPV, ABPV-KBV, and IAPV-KBV are 64%, 66% and 76% respectively (accessions; ABPV [Genbank: NC_002548.1], KBV [Genbank: NC_004807.1], IAPV [Genbank: NC_009025.1]). Based on multiple sequence alignments, we were able to find short regions that were highly conserved across the genomes of all three viruses.

RT-PCR Results

In total, the AKI primers were subjected to testing with 87 samples. Comparison of the RT-PCR results between the AKI primers and virus specific primers show that results are consistent and the AKI primers are effective. The results have been summarized in Table 2. Out of 87 samples, 49 samples were found to be positive using the AKI primers. Forty-seven of the positive samples gave a corresponding band for either ABPV, KBV and/or IAPV using specific primers. Forty-six, six and seven case of ABPV, KBV and IAPV were found respectively. This total of 59 suggests multiple infections. Two samples yielded a band with AKI primers, but did not give a band with any specific primers. The same two samples were later found to be ABPV based on sequence information from AKI amplicons. Out of 87 samples, 38 were found to be negative for the AKI primers. We detected no specific PCR product in any of these samples.

Of the 49 samples positive for AKI primers, 24 were positive for DWV and 11 for SBV. Of the 38 samples negative for AKI primers, 14 were positive for DWV and 4 for SBV. We conclude that the RNA extraction was successful, and the RNA was intact in the sample, but no template

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was available for the AKI or specific primers. In total, 39 DWV positives and 16 SBV positives were found.

Sequencing Results

Forty-eight samples were sequenced (21 AKI primers products, 13 ABPV, 5 KBV, 7 IAPV and 2 DWV) (as shown in parenthesis in Table 2 and Table 3, except for DWV). Four AKI sequences gave matching results to the sequence data obtained from the specific primer products.

However, three French samples and two Danish samples produced conflicting results with AKI and virus specific primers. The first French sample was found to be 100% identical to KBV based on the sequenced AKI product, but showed 95% identity to ABPV based on the sequenced ABPV product and 94% and 96% identity to IAPV using sequenced KBV and IAPV primer products respectively. The second and third French samples were found to be 100% identical to ABPV based on the sequenced AKI product, and showed highest fraction of identity to ABPV based on the sequenced ABPV product but highest identity to IAPV using sequenced KBV and IAPV primer products. Two separate extractions (1135 and 1138, Table 3) from one Danish sample were identical to ABPV using sequenced AKI primer products. Yet, another two separate extractions (1134 and 1140) from the same Danish sample were found to be 100% identical to IAPV using sequenced IAPV primer products.

We have identified three diagnostic positions in the AKI product which differentiate ABPV, KBV and IAPV (Fig. 1). An amplicon is produced by AKI primers in which only three positions vary consistently among ABPV, KBV and IAPV. Within the 100bp PCR fragment, position 49 (relative from the 5' end of forward AKI primer) is adenine in ABPV versus guanine in IAPV and KBV. Again, position 65 differentiates ABPV from IAPV and KBV with cytosine versus adenine respectively. Finally position 69 separates IAPV from ABPV and KBV with adenine versus guanine respectively. Two additional variable positions in the AKI product are not diagnostic. Although G in most genomes, position 32 was found to be A in certain ABPV AKI amplicons and complete reference genome [Genbank: NC_002548]. Position 52 is consistently T in most genomes, but was found to be C in the same above mentioned ABPV genome.

All AKI sequences were aligned and the diagnostic sites compared visually. All three diagnostic sites contained the expected combination of nucleotides. The virus identity could be verified for a few samples with missing data based on the remaining diagnostic sites (Fig. 1C). According to GenBank information, a non-diagnostic site at position 33 contains an A for ABPV [Genbank: NC_002548] instead of the G in most other APBV, and all KBV and IAPV sequences. Our results confirm this variation. Three Danish ABPVs were found to contain A at position 33. The second variable position (53) is T in most genomes, but was found to be C in ABPV complete genome [Genbank: NC_002548]. None of the ABPVs (n=18) in this study contained C at this position. (Refer Fig. 1C)

Detection of IAPV in Denmark

One of the samples from Denmark was found to be positive by AKI primers using RNA extracted from the head and this was identified as IAPV by using specific primers (Maori et al., 2009). Purified IAPV-integrated plasmid clone provided by Eyal Maori was used as positive control. To make a confirmation, the remaining bee was split into 4 thoracic and 4 abdominal fragments. RNA was extracted and RT-PCR was carried out separately from these sub-samples and four of the sub-samples were found to be positive for AKI primers (Tab. 3). These four samples were positive for IAPV using specific primers. Two of these 4 samples in addition tested positive using ABPV specific primers. Two of the four sequenced AKI primer products were found to be ABPV. Two products using IAPV specific primers were confirmed to be IAPV by sequencing. Both of these IAPV specific products (1140 and 1134 in Tab. 3) when aligned against respective genomes showed 100% identity to IAPV

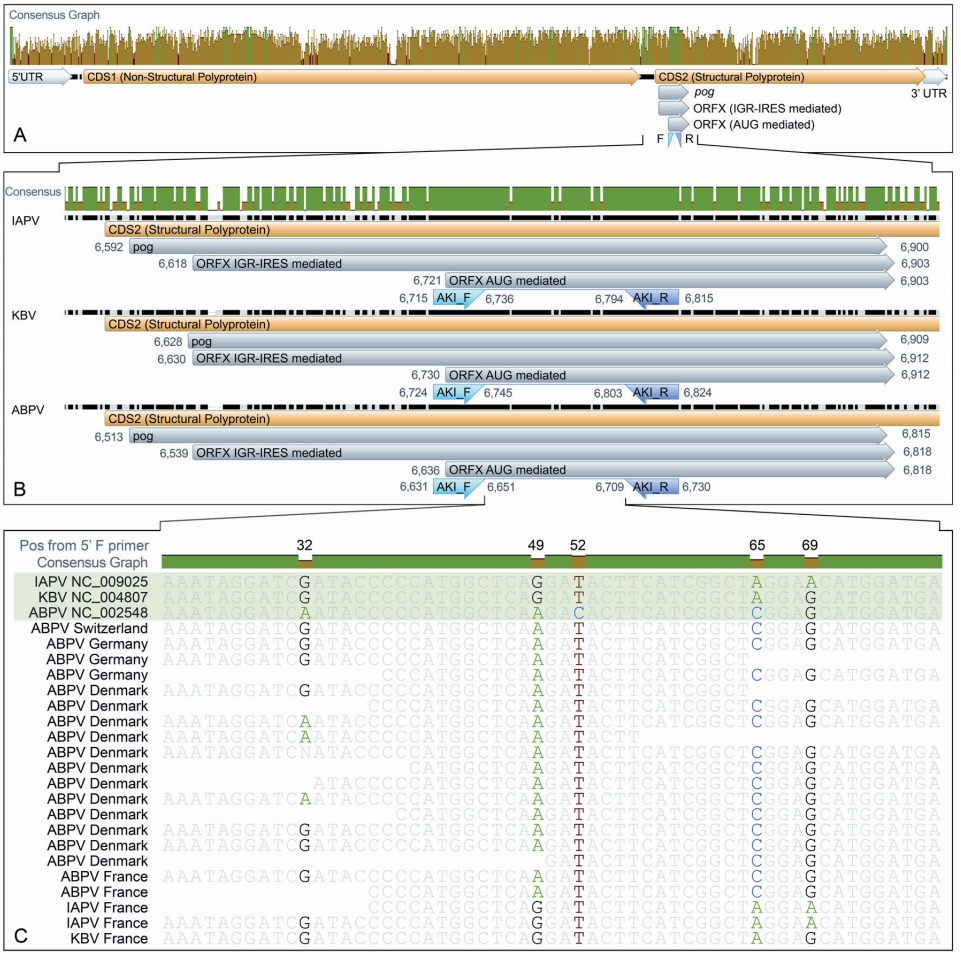

Fig. 1. (A) Consensus graph of the IAPV, KBV and ABPV genomes. Dark lines indicate 100% consensus while pale areas indicate less than 100% consensus. A schematic layout of a typical dicistroviral genome is depicted below showing the UTRs and the two coding ORFs. A third recently suggested positively selected ORF in the +1 reading frame named ORFX/pog (see text for details) with highly conserved regions is indicated. The position of forward and reverse AKI primers on the genome is also indicated. **(B)** Magnified region of the ORFX/pog region showing the highly conserved regions (dark on consensus graph and individual genomes) and position of CDS2 on each genome. The nucleotide position of ORFX (IGR-IRES mediated and AUG mediated) /pog and AKI primers on each genome (IAPV [Genbank: NC_009025], KBV [Genbank: NC_004807], ABPV [Genbank: NC_002548]) is indicated. **(C)** Magnified region of the AKI amplicon without primer region. The figure shows variable positions among the sequenced samples compared to reference genomes. Positions are counted from the 5' end of the forward primer. Image created using Geneious Pro 5.1.3 (Drummond et al., 2011).

[Genbank: NC_009025], but only 82% identity to KBV [Genbank: NC_004807] and 75% identity to ABPV [Genbank: NC_002548]. The sequence was also aligned to other IAPV complete genome accessions such as [Genbank: EF219380.1] (100% identity), [Genbank: EU436456.1] (96% identity), [Genbank: EU436455.1] (98% identity), [Genbank: EU436423.1] (94% identity). Further samples were tested from the same hive, however we did not detect any more positive cases. The IAPV affected colony survived the winter in good health.

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Table 2.

Summary of the results of this study. Total samples for each origin are indicated in square brackets. The number of samples obtained has been separated to positives and negatives based on results using the AKI primers. Section A - Number of samples producing a gel band for AKI primers and specific primers. Numbers in parenthesis indicate number of sequenced samples. Section B - Results of the sequenced samples. DNK-Denmark, CHE-Switzerland, DEU-Germany, FRA - France, TUR-Turkey, HUN-Hungary, ITA-Italy, MKD-Macedonia, ZAF-South Africa, BEN-Benin. (The Danish sample positive for IAPV is not included in Table 2, but in Table 3.)

Origin	Number of Samples	SECTION A				SECTION B		
		AKI primer	Specific primers			Sequence Results		
			ABPV	KBV	IAPV	ABPV	KBV	IAPV
DNK [48]	36 pos	36 (10)				10		
			36 (5)			5		
				1				
					0	0		
	12 neg	-	-	-	-			
CHE [3]	1 pos	1 (1)				1		
			1 (1)			1		
				-	1			
	2 neg	-	-	-	-			
DEU [5]	3 pos	3 (3)				3		
			1	-	-			
	2 neg	-	-	-	-			
FRA [5]	5 pos	5 (5)				2	1	2
			5 (4)			3		1
				5 (5)		0		5
					5 (5)			5
TUR [4]	2 pos	2	2 (2)			2		
	2 neg	-	-	-	-			
HUN [2]	1 pos	1	1 (1)	-	-	1		
	1 neg	-	-	-	-			
ITA [1]	1 neg	-	-	-	-			
MKD [3]	3 neg	-	-	-	-			
ZAF [8]	8 neg	-	-	-	-			
BEN [7]	7 neg	-	-	-	-			
Total	87	49	46	6	6	28	1	13

DISCUSSION

In Danish bee samples, ABPV, KBV and IAPV viruses are infrequent. Screening large samples in three separate reactions is expensive and time-consuming. Fast preliminary screening of samples can be done with the use of AKI RT-PCR primers, designed in a highly conserved region of IAPV, ABPV and KBV genomes. The primer sequences were determined to be 100% identical to all complete sequences, currently available for IAPV, KBV and IAPV on GenBank.

Acute Bee Paralysis Virus, KBV and IAPV have a typical dicistroviridae genome organisation consisting of a single positive stranded RNA strand containing two ORFs (Open Reading Frame) separated by an intergenic region (IGR) and flanked by UTRs (Untranslated regions)(Chen and Siede, 2007). The AKI primers have been designed in a highly conserved region of

Table 3.

Results for a single Danish bee sample infected with IAPV. 'x' indicate a positive result (bands on agarose gel electrophoresis). '-' indicates a negative result (no band on gel). Empty cells indicate 'not tested'. Text in parenthesis indicates the results of sequencing

	Fragment	Ext Num	AKI Primer	ABPV	KBV	IAPV	DWV	SBV
1	Head	1047	-	-	-	x	x	x
2	Thorax	1134	x	-	-	x (IAPV)		
3	Thorax	1135	x (ABPV)	x	-	x		
4	Thorax	1136	-	-	-	-		
5	Thorax	1137	-	-	-	-		
6	Abdomen	1138	x (ABPV)	x	-	x		
7	Abdomen	1139	-	-	-	-		
8	Abdomen	1140	x	-	-	x (IAPV)		
9	Abdomen	1141	-	-	-	-		

the CDS2 (Fig. 1). The region has been suggested to contain an overlapping gene in which the codons are shifted by a single base (+1), as compared to CDS2. This gene is predicted to be positively selected and has been named *pog* (predicted overlapping gene) by Sabath et al. (2009) and named ORFX by Firth et al. (2009) and Sabath et al. (2009). Firth et al. (2009), has further explained that the ORFX may be initiated at the IGR-IRES site or at an AUG start site which results in a considerable change in protein length. Therefore, the exact nucleotide position of the gene has not been confirmed. The possible nucleotide start and end positions of this newly predicted gene have been indicated in Fig. 1. The codon shifted overlapping gene may explain why all positions in this region are highly conserved across all three genomes.

Of 87 samples, 49 samples were found to be positive using the AKI primers described here. Out of 49 AKI primers positive samples, 46 gave a corresponding band in ABPV, KBV and/or IAPV using specific primers. In all, 46, six and seven cases of ABPV, KBV and IAPV were found respectively. Those two samples which gave a band with AKI primers, but did not give a band with any specific primers were confirmed to be ABPV based on sequence information. The explanation why these two samples failed to produce a band with specific primers may be due to degraded RNA, as the AKI primer product is only 101bp while specific product lengths are ABPV 452bp, KBV 414bp and IAPV 138bp. Out of 87 samples, 38 were found to be negative for the AKI primers, and we correspondingly detected no specific PCR product in any of these samples. Of the 49 samples positive for AKI primers, 24 were positive for DWV and 11 for SBV. Of the 38 samples negative for AKI primers, 14 were positive for DWV and 4 for SBV, indicating that the RNA extraction was successful, RNA was intact in these samples, but no template was available for the AKI or specific primers.

Forty eight samples were sequenced (Tab. 2). The sequences include 21 AKI primers products, 13 ABPV, 5 KBV, 7 IAPV and 2 DWV products. Almost all sequenced products gave expected results, except the following: one French sample tested positive using the ABPV 'specific' primers (Tentcheva et al., 2004), but was determined to be IAPV by sequencing. Similarly, all five French samples tested positive using the KBV 'specific' primers (Tentcheva et al., 2004), were identified

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as IAPVs by sequencing. The primer and genome sequence information in GenBank show that the ABPV and KBV primers used in this study can cross-react with the IAPV genome. This explains why some of the samples reported positive for ABPV and KBV were found to be IAPV by sequencing. However, based on AKI primer sequences for the same five French samples, we found one KBV, two IAPVs, and two ABPVs. Two of the DWV products from Germany were sequenced and confirmed to be DWV. Samples positive for DWV and SBV gave no products for the AKI primers validating absence of cross- reaction with these viruses.

The first case of IAPV in Denmark was identified during the period of this study. A single bee was found positive for IAPV using the head sample. The bee found positive for IAPV came from an experimental hive that had very high varroa numbers the previous year. As mentioned in the results, the rest of the bee was fragmented to 8 sub-samples. Of these, 4 samples gave bands for AKI primers and IAPV specific primers. Two sequenced AKI primers bands show ABPV while the 2 sequenced IAPV bands show IAPV. It is possible that the bee might have been infected with both viruses at the time of testing. Both of the sequenced products (1134 and 1140 in Tab. 3) when aligned showed 100% identity to IAPV [Genbank: NC_009025]. Out of the samples tested from the same bee hive, IAPV was only found in one adult bee. However, separate extractions from the same bee tested positive and the confirmation by sequencing makes the result reliable. This is the first record of IAPV in Denmark. In one former survey of honey bee viruses in Denmark, no test for IAPV was included (Nielsen et al., 2008). In the winter of 2007-2008, Danish beekeepers experienced high colony losses of 33%, but no IAPV was detected (Vejsnæs et al., 2010).

We conclude that the AKI primers are suited for the purpose of detection of the three viruses (ABPV, KBV and IAPV), from Danish samples as well as other European samples as far as we could test. Furthermore, we detected no false positives with the AKI primers, as the two samples found positive only with AKI primers were confirmed to be ABPV by sequencing. The very short fragment allows for the detection of RNA in cases where the RNA is degraded beyond the ability of detection using conventional specific primers.

CONCLUSION

The presence of ABPV, KBV or IAPV can be detected in a single RT-PCR assay using novel AKI primers thus reducing multiple PCR runs. This is an efficient way to reduce cost and time for the primary screening of multiple samples for the presence or absence of these three viruses if the incidence of the said viruses is low. Amplicon sequencing can further confirm AKI positives or specific PCR. The AKI primers are designed in a highly conserved region of the viral genome which may be responsible for critical viral processes.

ACKNOWLEDGEMENTS

This study was financed by Promilleafgiftsfonden, Faculty of Agricultural Sciences, Aarhus University and Dept. of Integrated Pest Management, Aarhus University. We wish to thank colleagues Eyal Maori, Cecilia Costa, Tamas Szalai, Hannelie Human, Olorounto Delphin Koudandé, Vincent Dietemann, Laurent Gauthier, and Aykut Kence who contributed the bee samples to be used in this study. We wish to also Steen Lykke Nielsen, Lene Hasmark Andersen, Trine Søberg Nielsen and Mogens Nicolaisen for their general and technical support.

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JEDNOCZESNE WYKRYWANIE WIRUSA OSTREGO PARALIŻU PSZCZÓŁ, KASZMIRSKIEGO WIRUSA PSZCZÓŁ I IZRAELSKIEGO WIRUSA OSTREGO PARALIŻU PSZCZÓŁ

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S t r e s z c z e n i e

Badanie pszczół na obecność wirusów jest zarówno czasochłonne, jak i drogie. Siedem wirusów, które są obecnie są najczęściej badane u pszczół, przy konwencjonalnym PCR wymagają przeprowadzenia oddzielnych analiz dla każdego z oznaczanych wirusów w badanej próbie. W pracy prezentowane jest wykorzystanie starterów AKI, przy pomocy których w pojedynczej reakcji można wykryć obecność każdego z trzech wirusów: wirus ostrego paraliżu pszczół (ABPV), wirus kaszmirski (KBV), izraelski wirus ostrego paraliżu (IAPV). Analiza sekwencji genów z GenBank wykazała, że wirusy te są ze sobą ściśle spokrewnione. W sekwencji wszystkich 3 wirusów został zidentyfikowany specyficzny region o 100% podobieństwie i opracowano startery, które pozwalają na powielenie tego regionu. Nowe startery zostały sprawdzone na 87 próbach. Jednocześnie wszystkie te próby zostały przebadane przy użyciu wcześniej stosowanych starterów, specyficznych dla każdego z wirusów. Stwierdzono, że we wszystkich przypadkach gdzie z użyciem specyficznego startera uzyskano produkt, ten sam produkt był również otrzymywany przy zastosowaniu starterów AKI. Uzyskane produkty AKI PCR zawierają tylko 101 par zasad, dzięki czemu są wystarczająco długie do wykrycia na żelu, ale na tyle krótkie, aby można je analizować za pomocą Real Time PCR.

Podczas badań, po raz pierwszy stwierdzono występowanie IAPV w Danii, co zostało potwierdzone poprzez sekwencjonowanie i otrzymanie specyficznego produktu z użyciem innych starterów.

Słowa kluczowe: wirusy pszczoły miodnej, IAPV, KBV, ABPV, startery AKI.