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Short Communication

Detection of honey bee (*Apis mellifera*) viruses with an oligonucleotide microarray

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ABSTRACT

In recent years, declines in honey bee (*Apis mellifera* L.) colonies have been observed to varying degrees worldwide with the worst losses in the USA being termed Colony Collapse Disorder (CCD). Pathogen load and the prevalence of honey bee viruses have been implicated in these losses and many diseased hives have multiple viruses present. We have designed and tested an oligonucleotide microarray which enables the simultaneous detection of nine honey bee viruses: *Acute bee paralysis virus, Black queen cell virus, Chronic bee paralysis virus, Deformed wing virus, Kashmir bee virus, Sacbrood virus, Israel acute paralysis virus, Varroa destructor virus 1* and *Slow paralysis virus.* The microarray can be used to robustly diagnose nine viruses in one test.

1. Introduction

Declines in honey bee (*Apis mellifera* L.) colonies in recent years have been partly attributed to the prevalence of viruses and pathogen load (Cox-Foster et al., 2007; vanEngelsdorp et al., 2009; Berthoud et al., 2010). At least 18 viruses have been reported to infect the honey bee *A. mellifera* L. (Allen and Ball, 1996; Ellis and Munn, 2005). To date, molecular methods applied to the detection of honey bee viruses have included conventional PCR (Benjeddou et al., 2001; Grabensteiner et al., 2007) and real-time PCR (Chantawannakul et al., 2006; Ward et al., 2007). However, the multiplex detection of more than two or three viruses can be difficult with PCR based methods and recent surveys have shown that up to five viruses can be present in a diseased hive (Berenyi et al., 2006). Oligonucleotide microarray techniques have been shown to detect multiple pathogens in a range of hosts (Wang et al., 2002; Boonham et al., 2003). We have designed and tested an oligonucleotide microarray which enables the simultaneous detection of nine honey bee viruses.

2. Materials and methods

Between 6 and 10, 50-mer oligonucleotide probes were designed for each of nine viruses: *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), *Deformed wing virus* (DWV), *Kashmir bee virus* (KBV), *Sacbrood virus* (SBV), *Israeli acute paralysis virus* (IAPV), *Varroa destructor virus 1* (VDV1) and *Slow paralysis virus* (SPV). In addition, four oligonucleotide probes were designed to the *A. mellifera* 18S rDNA gene to be used as positive controls and eight randomly generated nucleotide sequences were utilised as negative controls. Probe design was carried out using OligoArray 2.0 (Rouillard et al., 2003) with an additional BlastN search to check for cross-reactions between viruses (Altschul et al., 1997). The oligonucleotide probes chosen for printing onto the microarray are available as Supplementary material. Oligos were printed in triplicate onto glass slides (Austrian Institute of Technology) and stored in a dark, dry environment until use.

Infected material was produced by injecting bees with 1 μl of a preparation of purified virus diluted 1:20 in Ringer's saline solution (156 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$). Viral preparations were only available for the following viruses: BQCV, DWV, KBV, SBV, CBPV and IAPV. The bees were injected as white-eyed pupae and allowed to develop for a further 5 days before being sacrificed in liquid nitrogen prior to RNA extraction. Injected white-eyed pupae were extracted individually and naturally infected bees were extracted in groups of five individuals. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Crawley, UK) following the animal tissues protocol as supplied. The presence of each virus was confirmed using a suite of bee and virus specific TaqMan assays (Chantawannakul et al., 2006; Ward et al., 2007). Labelled cDNA was produced using the CyScribe first-strand cDNA synthesis kit (GE Healthcare, Bucks, UK). The labelled cDNA was then purified using a Qiagen PCR cleanup column (Qiagen, Crawley, UK) and eluted in 120 μl Hyb Buffer #1 (Ambion, Austin, USA). The purified cDNA was heated for 3 min at 95 °C, applied to the microarray and incubated overnight in a hybridisation chamber at 42 °C. Following hybridisation, each slide was washed initially in 2× SSC (150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS, followed by subsequent

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washes in 1× SSC and 0.1× SSC before being dried and scanned on a GenePix 4000B microarray scanner (Molecular Devices, USA).

3. Results and discussion

All the oligonucleotides designed were printed in triplicate onto glass slides and assessed for their specificity and relative sensitivity compared to other oligos designed for that virus. The oligonucleotides designed to ABPV, VDV1 and SPV were only assessed for cross-hybridisation of the other six viruses as positive material was not available. Fig. 1 demonstrates the performance of each oligonucleotide when labelled cDNA from bees infected with DWV, CBPV, BQCV, KBV, SBV and IAPV were applied to the microarray. The fluorescence values were normalised against an average of the fluorescence of the four 18S positive controls, which were positive in all samples hybridised to the array. No hybridisation to any of the bee virus oligonucleotides was detected when a healthy sample (i.e. real-time PCR negative for all viruses) was applied to the microarray.

The oligonucleotides designed for BQCV and KBV were highly specific for those viruses when labelled cDNA originating from bees infected with those viruses was applied to the microarray. Cross-hybridisations with oligonucleotides designed to other viruses were not observed (Fig. 1). Hybridisation of labelled DWV cDNA was successful for each of the DWV oligos. However, three cross-hybridisations were observed between DWV cDNA and oligos designed to other viruses. Two of the cross-hybridisations involved oligos designed to VDV1 (oligos VDV1-8 and VDV1-9). VDV1 has been shown to have 84% nucleotide identity to DWV (Ongus et al., 2004) and a further BLAST search of the two VDV1 oligos showed 100% homology to a recently sequenced isolate of DWV (GU109335) which was not available at the time the microarray was designed. The third cross-hybridisation was with an oligo designed to CBPV (CBPV-3). Homology was not observed to the currently sequenced isolates of CBPV (nor honeybee genome) and the DWV sample applied to the microarray had tested negative for CBPV by TaqMan, thus the cause of the cross-hybridisation with this oligonucleotide has not yet been determined.

Labelled cDNA originating from a bee infected with SBV hybridised to the SBV-specific oligos successfully. One of the oligos (SBV-2) demonstrated a lower level of fluorescence upon hybridisation of labelled cDNA. The sequence similarity of this oligo to known SBV sequences was not observed to be lower in the design process. Additionally, a cross-hybridisation was observed with an oligo designed for VDV1, VDV1-7, which had 100% homology to SBV in 46/50 nucleotides.

Only three of the oligos designed for CBPV were specific for the virus: CBPV-1, CBPV-5 and CBPV-7. When the array was designed, only 17 sequences of CBPV were available on Genbank, which may explain why five out of eight oligos did not demonstrate acceptable specificity. French isolates of CBPV have been shown to have up to 8.7% divergence (Blanchard et al., 2008) therefore the small number of isolates used to design the CBPV oligos may not have included the particular nucleotide variation present in the isolate of CBPV used to test the microarray. No cross-hybridisation was observed between labelled CBPV cDNA and oligos designed for other viruses.

Each of the oligos designed for IAPV were specific when labelled IAPV cDNA was applied to the microarray. One significant cross-hybridisation was observed to oligo BQCV-7. A further BlastN search (Altschul et al., 1997) was carried out and this oligo was found to have 100% homology to an IAPV sequence (EU224279) which was not available at the time the microarray was designed.

ABPV, KBV and IAPV have recently been described as members of the "ABPV complex" of closely related viruses (de Miranda et al.,

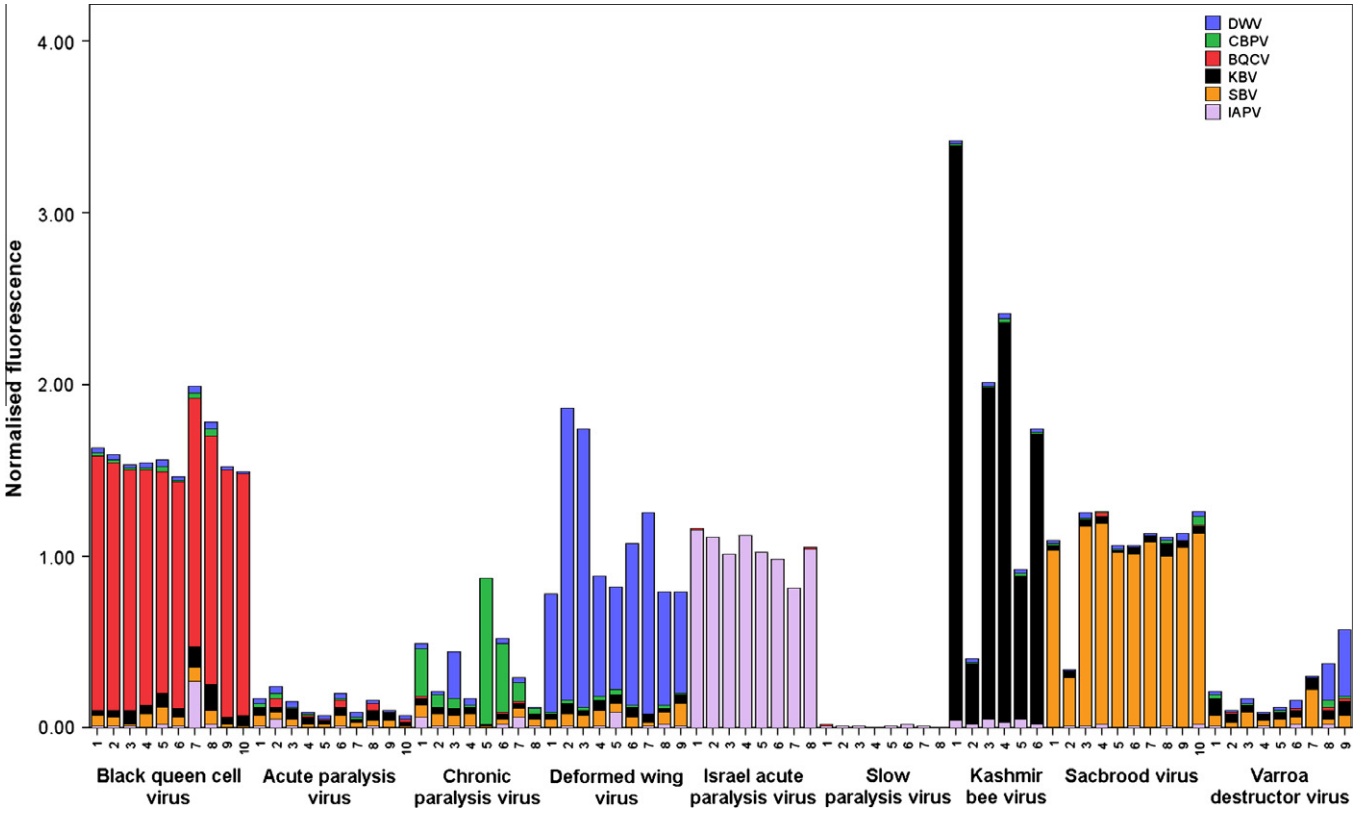

Fig. 1. Specificity of the oligonucleotides. Fluorescence levels for each oligonucleotide against six preparations of fluorescently labelled cDNA from bees infected with each of the viruses for which we had material (IAPV, SBV, KBV, BQCV, CBPV and DWV). Fluorescence levels were normalised against the average fluorescence level of the four positive control oligos.

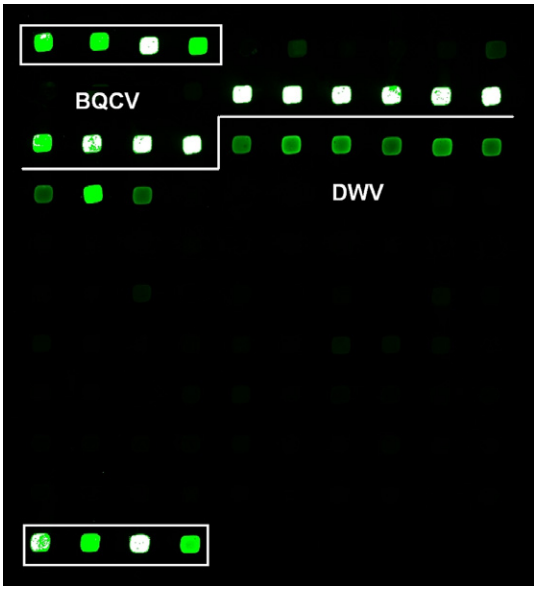

Fig. 2. The honey bee virus microarray demonstrating the multiplex detection of Black queen cell virus (10 spots above the line) and Deformed wing virus (nine spots below the line). The 18S controls are visible in the top and bottom left of the image (boxed).

2010). Whilst RNA was not available to directly test the ABPV oligos, the lack of cross reaction between ABPV, KBV and IAPV oligos when labelled cDNA from KBV and IAPV are applied to the array suggests that the array will be suitable for the discrimination of the three member viruses of this complex.

Overall, 40 of the 51 (78.5%) oligos designed demonstrated good specificity to the viruses they were designed to. By designing multiple oligonucleotides for each virus we have ensured that any cross-hybridising oligos can be excluded from future iterations of the microarray as a diagnostic test. A distinct advantage of microarrays in molecular diagnostics is the ability to detect multiple virus infections in one test. Fig. 2 demonstrates the ability of our microarray to detect a dual infection of BQCV and DWV – the 18S ribosomal controls can also be observed. A survey of hives in Austria (Berenyi et al., 2006) determined that most of the 90 diseased hives surveyed contained more than one virus and in northern Thailand co-infections of DWV, ABPV, SBV and KBV have been detected (Sanpa and Chantawannakul, 2009).

A comparison of the traditional screening method (TaqMan real-time PCR) with our microarray was carried out with two samples received for virus screening by the UK National Bee Unit. Both samples were tested with the suite of TaqMan assays described in Chantawannakul et al. (2006). Sample 1 was determined to be positive for DWV with an average Ct of 27.5 and Sample 2 positive for BQCV with an average Ct of 17.15. The samples were then hybridised to the separate microarrays and fluorescence determined. Fig. 3 shows the increased fluorescence for DWV and BQCV for

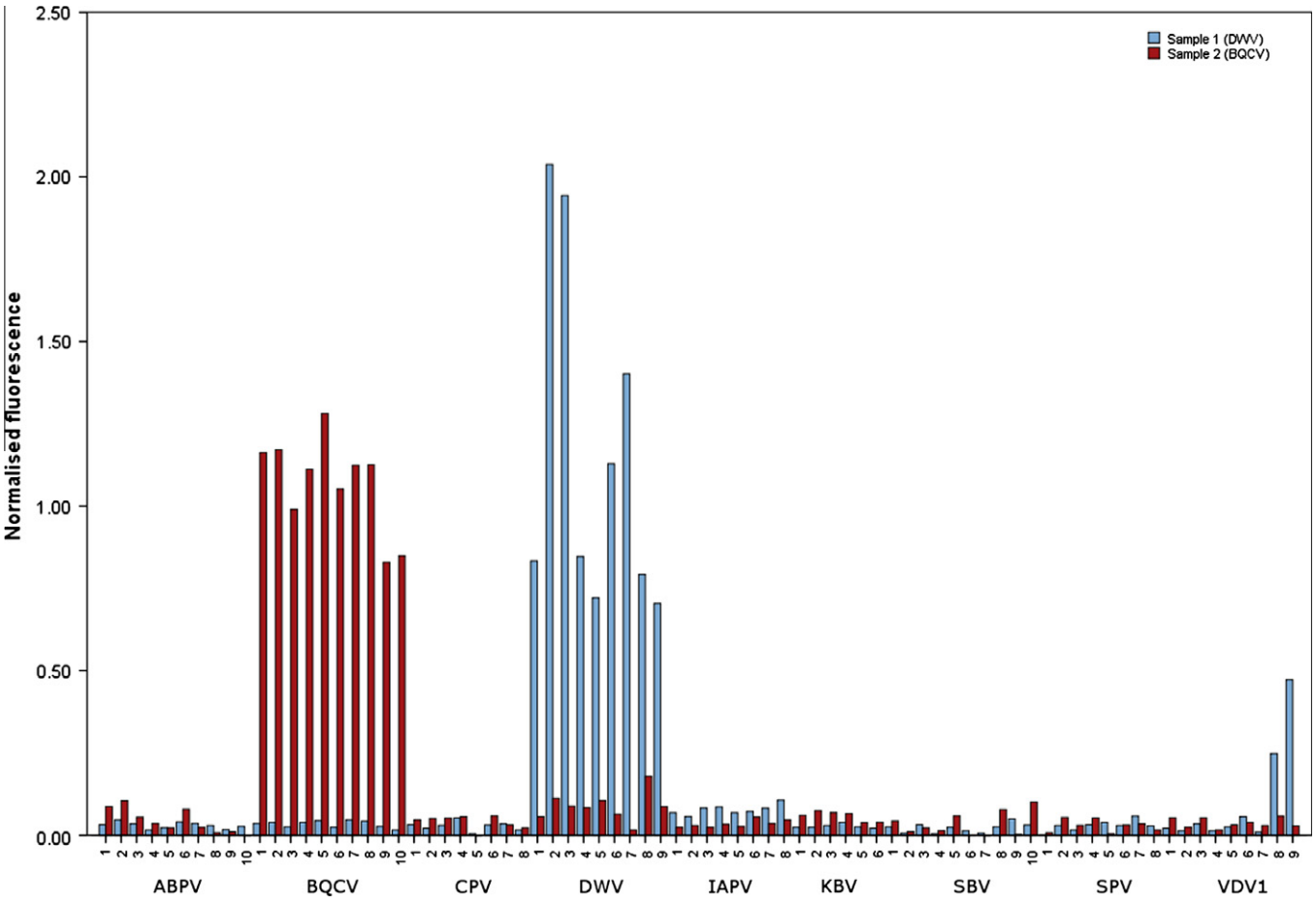

Fig. 3. Normalised fluorescence levels for two samples submitted for virus screening. Sample 1 (Blue) was positive for DWV by TaqMan (Avg. Ct = 27.5) and is shown to be positive by microarray. Sample 2 (Red) was positive for BQCV by TaqMan (Avg. Ct = 17.15) and also produced a positive diagnosis by microarray. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples 1 and 2, respectively. The cross-reactions between VDV1 oligos 8 and 9 and DWV are also reproduced in the DWV positive sample.

The 24–48 h required to proceed from extracted RNA to slide scanning and the requirement of expensive equipment to detect the fluorescence from hybridised cDNA currently inhibits the use of glass-slide microarray systems from routine diagnostic use. To overcome this, we are currently transferring the most promising oligos determined in this study to a new microarray format, Clondiag (www.clondiag.com), where all of the microarray steps are carried out in one tube using a precipitation staining reaction rather than fluorescence detection. The oligonucleotides designed in this study will allow a rapid and robust diagnostic test for nine bee viruses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jip.2011.03.004.

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