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Research notes

A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary

P. Chantawannakul [a,*], L. Ward [b], N. Boonham [b], M. Brown [b]

[a] *Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*
[b] *Central Science Laboratory, Sand Hutton, York, YO41 1LZ, UK*

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Abstract

Bee parasitic mite syndrome is a disease complex of colonies simultaneously infested with *Varroa destructor* mites and infected with viruses and accompanied by high mortality. By using real-time PCR (TaqMan), five out of seven bee viruses were detected in mite samples (*V. destructor*) collected from Thailand. Moreover, the results of this study provide an evidence for the co-existence of several bee viruses in a single mite. This is also the first report of bee viruses in mites from Thailand.

Keywords: *Varroa destructor*; Bee viruses; Disease transmission; *Apis mellifera*

Bee parasitic mite syndrome is a disease complex of colonies simultaneously infested with *Varroa destructor* mites and infected with viruses and accompanied by high mortality (Shimanuki et al., 1994). Eighteen viruses have been identified that are able to infect honeybees (Allen and Ball, 1996). Nucleotide sequences of eight of these viruses are documented and only four complete genome sequences are available on the EMBL sequence data [*V. destructor* virus 1, *Acute bee paralysis virus* (APV), *Kashmir bee virus* (KBV), *Sacbrood virus* (SBV)]. Most virus infection does not lead to clearly defined symptoms. The presence of bee viruses has traditionally been detected by using ELISA and more recently by conventional PCR methods. Bee viruses have often been reported to be associated with Varroa mite infestation. Mites collected from *Deformed wing virus* (DWV) infested bee colonies gave similar DWV ELISA optical density to those of the dead and deformed bees (Boven-Walker et al., 1999; Nordström, 2003), and gave positive reactions when using PCR methods (Chen et al., 2005; Tentcheva et al., 2004a). Similarly, KBV has been detected in mites collected from KBV positive colonies using specific primers in RT-PCRs (Chen et al., 2004). APV has also been detected in mites (Hung et al., 1996). Mites, therefore, have been suggested to play a potential important role as biological or mechanical vectors of bee viruses (Ball and Allen, 1988). More recent advances in PCR technology such as real-time PCR should lead to virus infection being detected more rapidly, at lower levels of infection and if necessary accurately quantified (Chen et al., 2005: Ward et al., 2005). TaqMan PCR was chosen for this study because this technology has many advantages over conventional PCR. The output from a real-time reaction is processed using standard calculations for all samples, thereby minimising user interpretation. Second, reaction tubes remain closed after the enzymic amplification of target, thereby minimising cross-contamination between samples. Third, real-time PCR can be fully quantitative. In addition, real-

* Corresponding author. Fax: +66 53 892259.
E-mail address: panuwan@yahoo.com (P. Chantawannakul).

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time testing is more rapid and less time consuming than conventional PCR tests that require post-PCR manipulations such as visualizing products on a gel. This factor is compounded when dealing with large numbers of samples. Finally, previous data has shown that TaqMan is up to 1000 times more sensitive than conventional PCR (Harju et al., 2005; Mumford et al., 2000; Ward et al., 2004).

In this report, we had chosen mite samples (*V. destructor*) collected from Thailand where mite infestation is the major problem for the bee industry and where the original hosts of mites and bee viruses, *Apis cerana* and *A. dorsata*, reside.

In this study, varroa mites found on developing honey bee (*Apis mellifera*) pupae were collected from an apiary in Lumphun province, Northern Thailand, in February 2005. RNA was extracted from the mites using the method described by Boonham et al. (2002). Briefly, individual mites were ground in a 0.5 ml microcentrifuge tube (using pellet grinders and matching tubes, Treff) with 50 μl of DEPC treated water and stored on ice. A slurry of 50% w:v of Chelex 100 resin (Bio-Rad) in water was added to each tube and heated at 94 °C for 5 min on a thermocycler. The extract was then centrifuged for 5 min at 13,000 rpm to pellet debris, and the supernatant was transferred to a fresh 0.6 ml micro-centrifuge tube and stored at −20 °C prior to use.

TaqMan forward and reverse primers and probes were designed for seven viruses, *Kashmir bee virus* (Accession AF263725), *Chronic bee paralysis virus* (CBPV) (Accession AF04230), *Acute paralysis virus* (Accession AF263733), *Deformed wing virus* (Accession NC004830), *Sacbrood virus* (SBV) (Accession NC002066), *Black queen cell virus* (BQCV) (Accession NC003784), and *Apis iridescent virus* (AIV) (Accession AF04230), using Primer Express software (Applied Biosystems, Branchburg, New Jersey, USA). The 5′-terminal reporter dye for each probe was 6-carboxyfluorescin (FAM) and the 3′ quencher was tetra-methylcarboxyrhodamine (TAMRA) (Table 1). The TaqMan assays were initially validated using pure viral cultures and virus infected bees (Ward et al., 2005). In addition to the assay designed for each virus, an internal positive control assay (IPC) was designed to the 16S rRNA mitochondrial gene of *V. destructor* (Accession AJ493124). An internal control was included in the study to compare extraction efficiencies between samples and to allow interpretation of negative results. Real-time PCRs were set up in 96-well reaction plates using Stratagene Brilliant Core reagents according to the manufacturer's protocol (Stratagene, La Jolla, California, USA). The reactions were set-up in 96-well plates using TaqMan. For each reaction, 1 μl of RNA extract was added to 24 μl of mastermix in the appropriate well giving a final reaction volume of 25 μl. Plates were cycled using generic system conditions (48 °C for

Table 1
Sequence of the TaqMan primers and probes designed for the detection of honey bee viruses and of an internal positive control for *Varroa destructor*

Primer/probe	Target	Sequence (5′–3′)
KBV83F	*Kashmir bee virus*	ACCAGGAAGTATTCCCATGGTAAG
KBV161R		TGGAGCTATGGTTCCGTTCAG
KBV109T		CCGCAGATAACTTAGGACATCAATCACA
APV95F	*Acute bee paralysis virus*	TCCTATATCGACGACGAAAGACAA
APV159R		GCGCTTTAATTCCATCCAATTGA
APV121T		TTTCCCCGGACTTGAC
CBPV304F	*Chronic bee paralysis virus*	TCTGGCTCTGTCTTCGCAAA
CBPV371R		GATACCGTCGTCACCCTCATG
CBPV325T		TGCCCACCAATAGTTGGCAGTCTGC
DWV958F	*Deformed wing virus*	CCTGGACAAGGTCTCGGTAGAA
DWV9711R		ATTCAGGACCCCACCCAAAT
DWV9627T		CATGCTCGAGGATTGGGTCGTCGT
AIV12F	*Apis iridescent virus*	GGCTAGTAAACGTAGTGGATATGACAAT
AIV106R		CACCTGGTGGTCCAAGAGAAG
AIV41T		TGATTGGAAATATATCTTCTTTAATAAACCCAGTTGCTCC
BQCV8195F	*Black queen cell virus*	GGTGCGGGAGATGATATGGA
BQCV8265R		GCCGTCTGAGATGCATGAATAC
BQCV8217T		TTTCCATCTTTATCGGTACGCCGCC
SBV311F	*Sacbrood virus*	AAGTTGGAGGCGCGYATTTG
SBV380R		CAAATGTCTTCTTACDAGAAGYAAGGATTG
SBV331T		CGGAGTGGAAAGAT
Varroa 16S 12290F	*Varroa destructor 16S rRNA*	GACTTACGTCGGTCTGAACTCAAA
Varroa 16S 12398R		TTGCGACCTCGATGTTGAATT
Varroa 16S 12330T		CAGATGAGCAATCTGCCTT

F, forward primer; R, reverse primer; T, probe. Probes consist of oligonucleotides with a 5′-reporter dye (FAM, 6-carboxy-fluorescein) and a 3′-quencher (TAMRA, tetra-methylcarboxyrhodamine).

Table 2
Twenty varroa mite samples collected from Thailand and mean C_T values of real-time PCR (TaqMan) using specific primers and probes for seven viruses as follows *Kashmir bee virus* (KBV), *Chronic bee paralysis virus* (CBPV), *Apis iridescent virus* (AIV), *Acute bee paralysis virus* (APV), *Deformed wing virus* (DWV), *Sacbrood virus* (SBV), *Black queen cell virus* (BQCV)

	Mean Ct values† (± SD)							
Sample number	KBV	CBPV	AIV	APV	SBV	DWV	BQCV	Internal control 16S rRNA
M1	34.608 (±0.077)	40	40	35.082 (±0.079)	35.670 (±0.146)	29.164 (±0.015)	37.466 (±0.151)	19.240 (±0.107)
M2	40	40	40	35.856 (±0.220)	33.352 (±0.398)	25.568 (±0.083)	36.999 (±0.165)	15.590 (±0.012)
M3	40	40	40	35.114 (±0.135)	32.981 (±0.050)	28.435 (±0.236)	29.775 (±0.308)	21.206 (±0.044)
M4	40	40	40	35.765 (±0.389)	25.244 (±0.035)	28.314 (±0.033)	33.360 (±0.062)	19.843 (±0.021)
M5	40	40	40	35.246 (±0.150)	24.234 (±0.001)	27.338 (±0.030)	31.961 (±0.180)	19.113 (±0.078)
M6	40	40	40	35.135 (±0.140)	23.303 (±0.014)	25.984 (±0.572)	22.696 (±0.191)	19.331 (±0.073)
M7	40	40	40	34.728 (±0.684)	40	26.365 (±0.301)	40	19.713 (±0.025)
M8	40	40	40	35.964 (±0.540)	33.294 (±0.032)	32.738 (±1.573)	40	19.019 (±0.059)
M9	37.575 (±1.174)	40	40	36.278 (±0.327)	31.917 (±0.223)	27.607 (±0.628)	40	19.585 (±0.087)
M10	40	40	40	35.094 (±0.263)	31.076 (±0.051)	26.798 (±0.515)	36.630 (±0.590)	23.927 (±0.096)
M11	40	40	40	36.002 (±0.297)	26.667 (±0.410)	29.026 (±0.923)	40	19.341 (±0.046)
M12	37.395 (±0.154)	40	40	36.302 (±0.109)	27.945 (±0.119)	29.199 (±0.522)	40	19.795 (±0.027)
M13	40	40	40	36.948 (±0.117)	24.076 (±0.598)	24.599 (±0.233)	40	20.411 (±0.016)
M14	40	40	40	35.347 (±0.239)	27.202 (±0.148)	26.776 (±0.384)	40	21.464 (±0.045)
M15	38.874 (±0.138)	40	40	37.407 (±0.629)	29.642 (±0.049)	33.920 (±0.227)	38.257 (±0.840)	22.409 (±0.033)
M16	36.932 (±0.899)	40	40	35.262 (±0.799)	32.121 (±0.068)	24.348 (±0.481)	28.375 (±0.269)	22.372 (±0.022)
M17	40	40	40	36.538 (±0.413)	30.008 (±0.170)	29.710 (±0.527)	40	18.807 (±0.005)
M18	40	40	40	36.472 (±0.177)	31.085 (±0.171)	28.552 (±0.624)	38.595 (±0.947)	20.706 (±0.038)
M19	40	40	40	36.275 (±0.342)	28.240 (±0.005)	24.060 (±0.266)	38.056 (±0.262)	21.579 (±0.034)
M20	40	40	40	33.8965 (±0.408)	31.942 (±0.049)	35.330 (0.746)	40	22.565 (±0.532)

All mite samples were also tested with the *V. destructor* 16S rRNA internal control to ensure edequate levels of RNA had been extracted. A C_T value recorded as 40 indicates negative results, whilst C_T values less than 40 indicate positive results.
*Colour shades indicate positive reactions. †Threshold cycles.

30 min, 95 °C for 10 min and 40 cycles of 60 °C for 1 min plus 95 °C for 15 s) within the 7900 Sequence Detection System (Applied Biosystems, Branchburg, New Jersey, USA) using real-time data collection.

The TaqMan results showed that a single mite was carrying up to five viruses, the most prevalent being KBV, APV, DWV, SBV, and BQCV (Table 2). TaqMan C_T values for KBV, APV, DWV, BQC and were in mainly high

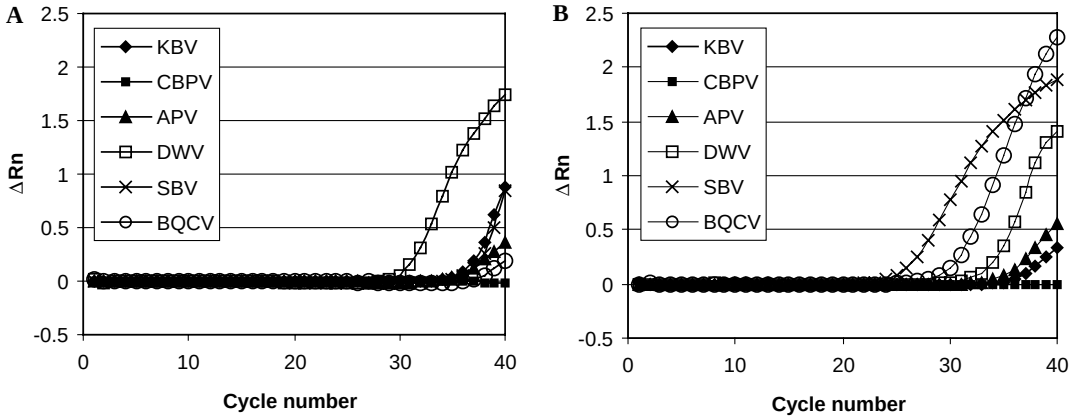

Fig. 1. Amplification plots following real-time PCR demonstrating the detection of viruses in individual mites. (A) Sample M1 and (B) sample M16, illustrating the detection of the five bee viruses DWV, KBV, SBV, APV, and BQCV. Single replicates are shown for clarity, but each sample was tested in duplicate.

values (above C_T 30) indicating that the levels of these viruses were low in the mites. In contrast, the C_T values for SBV and DWV were lower, mainly between C_T 20 and 31, indicating higher levels of these two viruses (Figs. 1A and B). Nevertheless, when collecting the samples, the apiary showed no sign of diseases or colony collapse.

This is the first preliminary study and report of bee viruses in varroa mites in Thailand. The results of this study provide further evidence for the co-existence of several bee viruses in a single mite that have potential to cause multiple infections in a bee colony. For example, in the case of APV and KBV, which were reported in one US honeybee colony (Hung et al., 1996). Ninety-two percent of the apiaries in France were found positive for at least three different viruses (31% of the apiaries contained three viruses, 36% contained four viruses, and 25% contained five viruses) following a survey in 2002 (Tentcheva et al., 2004b). The questions raised are whether all viruses are capable of replication in mites and, importantly, how the viruses may compete or suppress each other to exhibit different abilities to transmit and cause disease in honeybees. This study also shows that real-time PCR is a powerful tool for studying virus infection of mites and honeybees especially at the "inapparent" or covert infection stage. Indeed, in this study TaqMan was able to detect virus levels (higher than C_T 30) that would be below the detection sensitivity of conventional RT-PCR. The quantitative aspects of the technique will also help to clarify the relationship between viruses, mites, and honey bees.

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