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Research article

The ectoparasitic mite *Tropilaelaps mercedesae* (Acari, Laelapidae) as a vector of honeybee viruses

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Abstract. The ectoparasitic mites *Varroa destructor* and *Tropilaelaps mercedesae* share life history traits and both infect honeybee colonies, *Apis mellifera.* Since *V. destructor* is a biological vector of several honeybee viruses, we here test whether *T. mercedesae* can also be infected and enable virus replication. In Kunming (China), workers and *T. mercedesae* mites were sampled from three *A. mellifera* colonies, where workers were exhibiting clinical symptoms of deformed wing virus (DWV). We analysed a pooled bee sample (15 workers) and 29 mites for the presence of Deformed wing virus (DWV), Black queen cell virus (BQCV), Sacbrood virus (SBV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV), and Chronic bee paralysis virus (CBPV). Virus positive samples were analysed with a qPCR. Only DWV +RNA was found but with a high titre of up to 10^8 equivalent virus copies per mite and 10^6 per bee. Moreover, in all DWV positive mites (N= 12) and in the bee sample virus –RNA was also detected using RT-PCR and tagged RT-PCR, strongly suggesting virus replication. Our data show for the first time that *T. mercedesae* may be a biological vector of DWV, which would open a novel route of virus spread in *A. mellifera.*

Keywords: Apis mellifera, honeybees, *Tropilaelaps mercedesae*, vector, viruses.

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Introduction

Mites of the genus *Tropilaelaps* (Acari: Laelapidae) are ectoparasites of honeybees *Apis* native to Asia (Delfinado and Baker, 1961; Laigo and Morse, 1968). The primary host of one of the better known species *Tropilaelaps clareae* is *Apis dorsata* (Laigo and Morse, 1968) but *Tropilaelaps* mites were able to switch to the western honeybee, *Apis mellifera* (Delfinado and Baker, 1961; Anderson and Morgan, 2007). *Tropilaelaps clareae* was first discovered on *A. mellifera* in the Philippines (Delfinado and Baker, 1961). *Tropilaelaps* species seem to be prevalent in Asia and are able to infect a wide spectrum of honeybee species raging from *Apis mellifera, A. cerana, A. dorsata, A. florae* and *A. laboriosa* (Bailey and Ball, 1991; Schmid-Hempel, 1998). However, these mites appear to be particularly pathogenic in *A. mellifera* (Burgett et al., 1983; de Jong et al., 1982; Laigo and Morse, 1969). Similar to *Varroa destructor* Anderson and Trueman (Acari: Varroaidae), *Tropilaelaps* mites are infecting brood and suck haemolymoph. Up to four female mites can invade the same brood cell (Burgett and Akratanakul, 1985; de Jong et al., 1982).

Given that both mite genera are sucking haemolymph, it seems likely that they obtain pathogens from their hosts and may act e.g. as vectors of viruses, because mites switch between different host individuals (Delfinado-Baker and Aggarwal, 1987; Sammataro et al., 2000). Indeed, several honeybee viruses have been found in *V. destructor* like Deformed wing virus (DWV), Black queen cell virus (BQCV), Sacbrood virus (SBV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV) and Chronic bee paralysis virus (CBPV; Chen and Siede, 2007). Moreover, DWV is replicating in *V. destructor* (Yue and

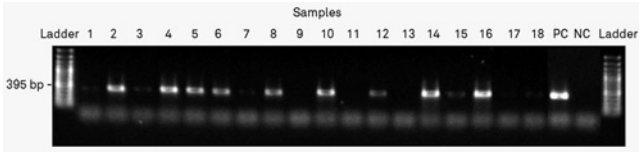

Figure 1. Results of the qualitative PCR for DWV +RNA (Ladder = size marker [100 bp peqlab], PC = positive control, NC = negative control). 18 samples are shown. In total, 12 out of 23 mites were positive for DWV +RNA.

Genersch, 2005), despite considerable taxonomic distance between host and parasite.

Here we hypothesise that honeybee viruses can also infect and replicate in *Tropilaelaps* spp. If this is the case, *Tropilaelaps* mites could also act as biological vectors similar to the other ectoparasitic mite *V. destructor* (Chen and Siede, 2007). Here we test this hypothesis by analyzing *T. mercedesae* (Anderson and Morgan, 2007) for the presence and replication of six honeybee viruses (KBV, SBV, CBPV, BQCV, ABPV, DWV).

Materials and methods

Honeybee workers and *T. mercedesae* mites were sampled from three *A. mellifera* field colonies in Kunming (China), where adult workers were exhibiting clinical DWV symptoms (= deformed wings, Chen and Siede, 2007). These colonies were screened at a weekly basis for the presence of *Varroa destructor* using standard methods (sticky bottom boards for natural mite fall and bee samples from the brood nest; cf. Sammataro et al., 2000) for two months prior to and also during the sampling. The samples were suspended in the RNA stabilising agent RNAlater® (Ambion) and stored at -20°C before RNA extraction.

Sample preparation

RNA extraction of individual *T. mercedesae* mites (N= 23) and the pooled worker sample (N= 15 bees) was performed using routine protocols (Tentcheva et al., 2004) with the kit NucleoSpin® RNA II (Macherey-Nagel, elution volume 60 µl). Then, the RNA was retrotranscribed in cDNA at 25°C for 10 min and at 37°C for 50 min with the M-MLV RT Thermoscript® RT-PCR (Invitrogen) using random hexamers. The cDNA was diluted 10 fold and stored at -20°C. We used $5x10^7$ copies of Tobacco Mosaic Virus as an exogenous internal reference RNA.

PCR analysis

Qualitative analysis for virus +RNA

The assays for the qualitative PCR of the six +RNA honeybee viruses (KBV, SBV, CBPV, BQCV, ABPV, DWV) were performed using routine protocols and published primers (Tentcheva et al., 2004, 2006) with the following modifications: two µl of cDNA were mixed with 2 µl 10x PCR buffer, 0.4 µl dNTP's 10 mM, each primer at a final concentration of 0.4 µM, and 0.16 µl of *Taq* polymerase 5U / µl (AmpliTaq® Gold); the final volume was 20 µl. We used the GeneAmp® PCR system 2700 with the following profile: 95°C for 5 min then 35 cycles of 30 s at 94°C, 30 s at 56°C, 45 s at 72°C and then a final step of 7 min at 72°C. The PCR products were then visualised on 1.5 % Agarose gels stained with GelRed.

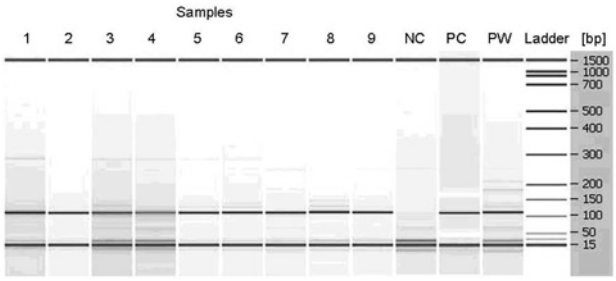

Figure 2. Results of the tagged primer PCR for the detection of DWV –RNA (NC = negative control, PC = positive control, PW = Pooled Workers, Ladder = size marker 100 bp peqlab). The fragments 15 bp and 1500 bp are internal size markers. All samples were positive (105 bp fragment).

Quantitative analysis for virus +RNA

For the DWV positive samples, we also quantified virus loads using qPCR and routine methods (Tentcheva et al., 2004, 2006, Gauthier et al., 2007). However, the initial amplification attempts with the published primers for qPCR failed. Therefore, we cloned with standard methods (Sambrook and Russell, 2001) the amplification products (395 bp) of our positive DWV samples from the qualitative analysis in a pGEM®-T easy (Promega) following the supplier's recommendation. We sequenced the clone (FASTERIS SA, Genève, Switzerland) and designed new primers for the qPCR using Sequencher TM (fwd 5'-TGGAATGCGTCCCGAACT-3' and

rev 5'-AATAAAACCTCACACTATATTCACGGATT-3'). Then, we amplified the samples with the new primer set using qPCR SybrGreen I no Rox and the protocols of the supplier (Kit Eurogentec) under the following conditions: 6 µl qPCR Mastermix 2x Eurogentec, 3 µl H2O, each primer at a final concentration of 300 nM, 2 µl of cDNA for a final volume of 12 µl. The used temperature profile was 50°C for 2 min, 95°C for 10 min, 40 cycles 95°C for 15 sec and 60°C for 1 min, 60°C dissociation and a melt curve with ramping from 60°C to 95°C rising from 1°C each step, waiting the first step 45 sec, and 5 sec for each steps afterwards.

Qualitative analysis for virus –RNA

For detection of the strand specific –RNA, which is the matrix for replication of +RNA viruses, we used established methods (Yue and Genersch, 2005) with modifications for a two step RT-PCR (see above). We used the M-MLV RT Thermoscript® RT-PCR under the same conditions as above but changing the 37°C step to 55°C for 50 min with our new primer set in a tagged system in order to increase the strand-specific diagnosis of DWV –RNA. Then, a 2nd PCR was performed using the forward primer corresponding to the tag sequence or the reverse primer. The PCR products were then visualised with the Agilent 2100 Bionanalyser® DNA 1000 kit following the supplier's recommendations.

Results

No *V. destructor* mites were found in any of the three sampled colonies. All samples were negative for five viruses (KBV, SBV, CBPV, BQCV and ABPV). However, the pooled worker sample and 12 out of 23 *T. mercedesae* mites were positive for both +RNA (Fig. 1) and –RNA of DWV (example of 9 mites, Fig. 2). The qPCR (efficiency ~98 % and detection level 10^2) revealed from 10^3 up to 10^8 equivalent DWV virus copies per individual mite and

10^6 equivalent copies for an individual honeybee worker (based on the pooled sample).

Discussion

Our data show for the first time that the honeybee virus DWV can infect *T. mercedesae* and replicate in this host. This is also the first evidence for a honeybee virus in another Acari host other than *V. destructor.* In light of the similar life histories of the ectoparasitic honeybee mites *V. destructor* and *T. mercedesae*, this strongly suggests that *T. mercedesae* may also act as a biological vector of honeybee viruses. Indeed, our laboratory data give strong support to earlier field observations (Burgett et al., 1983). Burgett et al. (1983) reported of a correlation between infestation rates of honeybee brood with *Tropilaelaps* mites and adult honeybee workers with deformed wings (one clinical symptom of DWV infections, Chen and Siede, 2007).

The need for new qPCR primers for our tested Kunming population of DWV suggests considerable genetic variation between virus populations, which is not surprising in light of the high mutation rates of +RNA viruses (Domingo and Holland, 1997). Up to 10^8 equivalent virus copies were found per *T. mercedesae* mite, which is common in *V. destructor* (Tentcheva et al., 2006). In general, the DWV loads in the tested worker and *T. mercedesae* mite samples are within the reported range found for European *A. mellifera* and *V. destructor* (Tentcheva et al., 2006), strongly suggesting that DWV can replicate in both mites. Indeed, similar to previous findings for *V. destructor* (Yue and Genersch, 2005) we also found -RNA of DWV in *T. mercedesae*, which is a clear sign of replication for +RNA viruses. Similarly, DWV was also found in bumble bees (*Bombus terrestris* and *Bombus pascuorum*, Genersch et al., 2006). Moreover, CBPV was detected in ants (*Camponotus vagus* and *Formica rufa,* Celle et al., 2008) and KBV in the German wasp (*Vespula germanica*, Anderson, 1991). The observation that the mite *V. destructor* is a biological vector of DWV (Yue and Genersch, 2005) and infections of several social insect hosts by honeybee viruses underlines the potential of +RNA viruses to persist in the environment as generalists. This is in line with the high mutation rates of RNA viruses (Domingo and Holland, 1997) and previous predications that the consequent genetic variants should be more likely generalists (Woolhouse et al., 2001). In light of these previous findings, our detection of DWV replication in *T. mercedesae* strongly supports the view that honeybee +RNA viruses are able to expand to multiple hosts, including other social insect species and their associated ectoparasites. Viruses with RNA genomes are in general of special concern, because of their great capacity for change and their exploitation of new opportunities (Scott et al., 1994). Indeed, they are among the most mutable of all parasites (Scott et al., 1994). This indicates that the amount and role of shared pathogens has been considerably underestimated in the social insects, which could be relevant given the potential effects of host shifts for virulence (Woolhouse et al., 2001). Indeed, a potentially higher DWV virulence in bumble bees than in honeybees has already been reported (Genersch et al., 2006). The possible impact of *Tropilaelaps* mites on the virulence of the honeybee viruses and their potential role for honeybee colony losses should be evaluated in future studies. In any case, the combination of our laboratory data with earlier field observations (Burgett et al., 1983) strongly suggest that *Tropilaelaps* mites may be biological vectors of honeybee viruses, thereby constituting another transmission route both within and between honeybee colonies.

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