Journal of Invertebrate Pathology 134 (2016) 6–11

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Varroa destructor Macula-like virus, Lake Sinai virus and other new RNA viruses in wild bumblebee hosts (*Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*)

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ARTICLE INFO

Article history:
Received 28 May 2015
Revised 3 November 2015
Accepted 7 December 2015
Available online 18 December 2015

Keywords:
Bombus pascuorum
Bombus lapidarius
Bombus pratorum
Pathogen
Varroa destructor Macula-like virus
Lake Sinai virus
Slow bee paralysis virus
Big Sioux river virus

ABSTRACT

Pollinators such as bumblebees (*Bombus* spp.) are in decline worldwide which poses a threat not only for ecosystem biodiversity but also to human crop production services. One main cause of pollinator decline may be the infection and transmission of diseases including RNA viruses. Recently, new viruses have been discovered in honeybees, but information on the presence of these in wild bumblebees is largely not available.

In this study, we investigated the prevalence of new RNA viruses in *Bombus* species, and can report for the first time *Varroa destructor* Macula-like virus (VdMLV) and Lake Sinai virus (LSV) infection in multiple wild bumblebee hosts of *Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*. We sampled in 4 locations in Flanders, Belgium. Besides, we confirmed Slow bee paralysis virus (SBPV) in wild bumblebees, but no positive samples were obtained for Big Sioux river virus (BSRV). Secondly, we screened for the influence of apiaries on the prevalence of these viruses. Our results indicated a location effect for the prevalence of VdMLV in *Bombus* species, with a higher prevalence in the proximity of honeybee apiaries mainly observed in one location. For LSV, the prevalence was not different in the proximity or at a 1.5 km-distance of apiaries, but we reported a different isolate with similarities to LSV-2 and "LSV-clade A" as described by Ravoet et al. (2015), which was detected both in *Apis mellifera* and *Bombus* species. In general, our results indicate the existence of a disease pool of new viruses that seems to be associated to a broad range of *Apoidae* hosts, including multiple *Bombus* species.

1. Introduction

Bumblebees are regarded as important pollinators of wild flora (Goulson, 2010; Goulson and Darvill, 2004) and of many crops including tomatoes, cucumbers and other top fruit (Velthuis and van Doorn, 2006). Hymenopteran pollinators such as honeybees and wild bumblebees forage for nectar and pollen from a wide range of plants as food sources (Goulson, 2010; Hagbery and Nieh, 2012; Hennig and Ghazoul, 2012). Despite their importance, pollinators are declining worldwide (Ghazoul, 2005; Potts et al., 2010). In the last decades, different RNA viruses have been described in honeybees, such as Black queen cell virus (BQCV) (Peng et al., 2011), Deformed wing virus (DWV) (Evison et al., 2012; Genersch et al., 2006), Sacbrood virus (SBV) (Reynaldi et al., 2014; Singh et al., 2010) and Slow bee paralysis virus (SBPV) (Bailey and Woods, 1974). However, these so-called honeybee pathogens have recently also been reported in solitary bees (Ravoet et al., 2014), bumblebees (Fürst et al., 2014; McMahon et al., 2015), and also in non-*Apoidae* hosts as *Vespula* species (Evison et al., 2012).

Because some important pollinators including honeybees and bumblebees are polylectic foragers, they share common food plants (Rohde et al., 2013; Singh et al., 2010). Therefore, a possible indirect transmission route of pathogens for different bee taxa has been described by means of contact with shared contaminated flowers (Singh et al., 2010). Besides, a bi-directional transmission between honeybees and bumblebees remains possible (Fürst et al., 2014; McMahon et al., 2015). Probably these RNA-viruses share multiple pollinator hosts, pointing to an interconnected network of RNA viruses within and among a range of pollinator species (Fürst et al., 2014; McMahon et al., 2015). It therefore seems that these RNA viruses pose a threat for different *Apoidae*

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http://dx.doi.org/10.1016/j.jip.2015.12.003
0022-2011/

species and other members of the pollinator community (McMahon et al., 2015).

Studies performed to elucidate the decline in honeybee colonies (Granberg et al., 2013; Runckel et al., 2011) has resulted in the discovery of new viruses, including Lake Sinai virus (LSV) (Runckel et al., 2011), Big Sioux river virus (BSRV) (Runckel et al., 2011), SBPV (Bailey and Woods, 1974; de Miranda et al., 2010) and *Varroa destructor* Macula-like virus (VdMLV) (de Miranda et al., 2011). Recently, these viruses have also been found in solitary bees (Ravoet et al., 2014), but none, except SBPV, have been reported in European bumblebees. Despite that RNA viruses seem to be generally present in Hymenopteran hosts, data on new RNA viruses related to widespread pollinators as bumblebees are still lacking.

In this study, our first aim was to screen whether VdMLV, LSV, BRSV and SBPV are present in wild bumblebee species, and secondly to investigate on the effect of distance to honeybee apiaries on the prevalence of these RNA viruses in bumblebees. Therefore, we selected four locations in the provinces of East- and West-Flanders (Belgium) and sampled wild bumblebees in the close proximity and at a distance of 1.5 km from an apiary of honeybees (*Apis mellifera*). Selecting three abundant species for wild bumblebees, we focused on *B. pascuorum*, *B. lapidaries* and *B. pratorum*.

2. Materials and methods

2.1. Defining study sites close to an apiary (Api-near) and at a distance (Api-far)

We sampled bumblebees in the provinces of East- and West-Flanders (Belgium) in 2013, each containing two coupled study sites (4×2 study sites in total) (Supplementary Fig. S1). We designed our locations to have a study site close to an apiary (Api-near) and at a 1.5 km-distance (Api-far). The choice of an Api-near site was made based on the distribution of apiaries in Flanders, Belgium. A distribution map of beekeepers (registered at the Federal Agency for the Safety of the Food Chain, Brussels, Belgium) and verified by contacting the beekeepers, was used to generate an actual distribution map of beekeepers and actual locations of honeybee hives. This map was used to pinpoint the Api-near sites (screened to be rich in apiaries) and to search for the Api-far study sites, with a minimum number of apiaries in the neighborhood. We selected a radius of 750 m as maximum forage distance, as described previously (Parmentier et al., 2014) to define a distance of 1.5 km between the Api-near and Api-far study sites. Around the Api-near sites we counted a mean of 6.5 ± 2.6 honeybee hives per km^2 compared to 0 ± 0 for the Api-far sites. In Belgium the mean number of honeybee hives per km^2 is 3.6 (Chauzat et al., 2013), which is in between our two extremes (see Table 1).

2.2. Sample collection and RNA extraction

In all locations we sampled individual *B. pascuorum*, *B. lapidarius* and *B. pratorum* in equal numbers per study site (Api-near and Api-far), with a total of 28, 80 and 18 samples per species and per location, respectively. Simultaneously in the Api-near sites, we sampled honeybees in 26 hives. We obtained samples of 30 randomly selected bees per hive. As representative bumblebee species, we selected *B. pascuorum*, *B. lapidarius* and *B. pratorum* because they were generally present in our study sites. Caught bumblebees were transferred in individual tubes which were put immediately on dry ice and stored at −70 °C at the end of the sampling day. Honeybees were pooled per 30 whereas bumblebees were crushed individually for 5 min after adding 4 ml or 700 µl Qiazol® (Qiagen Benelux, Venlo, the Netherlands), respectively, and zirconia (0.1 mm) and stainless steel (1 mm) beads. A total of 500 µl of supernatants was centrifuged at 17000*g* for 3 min. Next, 900 µl of Qiazol was added to 100 µl supernatants and the protocol was followed according to manufacturer's instructions (RNeasy Lipid Tissue; Qiagen Benelux, Venlo, the Netherlands). The RNA was eluted from the column in 50 µl RNA-free water. Honeybees sampled from each hive were pooled in groups of 10 honeybees and this was done in triplicate. In each tube 4 ml Qiazol® was added for bead beating and further processed as described for individual bumblebee samples.

2.3. MLPA analysis and reverse transcriptase PCR

Initially, we screened bumblebee and honeybee samples for a range of known positive-sense single stranded RNA viruses, by employing multiple ligation-dependent probe amplification (MLPA) using the RT-MLPA kit (MRC-Holland, Amsterdam, the Netherlands) The MLPA technique is capable to detect multiple viruses at once with only a minor loss of sensitivity compared to strand-specific PCRs (De Smet et al., 2012; de Miranda et al., 2013). We used probes designed for the detection of positive-sense single-stranded RNA of the following four viruses: VdMLV, LSV, SBPV, and BSRV (De Smet et al., 2012; Ravoet et al., 2014). The composite probes contained aside from the virus specific part, a stiffer region to differentiate the length of the probes and a primer region to amplify the probes. Probe amplification was performed by 5′ FAM-labeled primers. Fragments were separated by capillary electrophoresis and aligned using an intern standard (GS500 LIZ). Fragment detection was achieved by a calibrated fluorochrome reader (Genetic service Unit UZ Ghent, Ghent University, Ghent, Belgium) and sample processing by employing the Peak Scanner vs. 2 software, selecting option "Sizing default – Primer present".

For sequence analysis, positive MLPA samples were selected and further used. Reverse transcriptase was performed on initial RNA with random hexamer primers with the Revert AidTM First Strand cDNA Synthesis Kit (Invitrogen, Merelbeke, Belgium). All strand-specific PCR reactions contained: 1.5 mM $MgCl_2$; 0.2 mM dNTP; 1.25 U Hotstar Taq DNA polymerase (Qiagen), 1–5 µl cDNA product (300–500 nmol of RNA) and 2 µM of each primer. We used specific primers described by Ravoet et al. (2015) for LSV BRSV, SBPV and VdMLV. The following cycling conditions were used:

Table 1
Presence of RNA viruses detected in wild bumblebees (*Bombus* sp.) in the proximity of apiaries (Api-near) and at a 1.5 km distance (Api-far).[a]

Bombus sp.	VdMLV		LSV		SBPV		BRSV	
	Api-near	Api-far	Api-near	Api-far	Api-near	Api-far	Api-near	Api-far
B. lapidarius	(14; 1)	(14; 0)	(14; 1)	(14; 0)	(14; 0)	(14; 1)	(14; 0)	(14; 0)
B. pascuorum	(40; 10)	(40; 3)	(40; 14)	(40; 12)	(40; 3)	(40; 1)	(40; 0)	(40; 0)
B. pratorum	(9; 0)	(6; 1)	(9; 0)	(6; 0)	(9; 0)	(6; 0)	(9; 0)	(6; 0)
Total Bombus sp.	(63; 11)	(60; 4)	(63; 14)	(60; 12)	(63; 3)	(60; 2)	(63; 0)	(60; 0)
Statistics Api-near vs. Api-far*	**0.011**		**0.67**		**0.76**		–	

[a] Results are presented as (sample number; number positives); * *P*-value, $\alpha = 0.05$, GLM.