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ScienceDirect
Journal of Invertebrate Pathology 98 (2008) 239–242
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Short Communication

Occurrence and genetic analysis of picorna-like viruses infecting worker bees of *Apis mellifera* L. populations in Devon, South West England

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Received 8 October 2007; accepted 13 February 2008
Available online 21 February 2008

Abstract

Viruses of the European honeybee, *Apis mellifera* L. are known to reside at low levels in colonies, typically showing no apparent signs of infection. Using reverse transcription-PCR (RT-PCR), 23 apiaries in Devon were screened for the presence of 6 honeybee viruses, with positive colonies being analysed for viral genetic diversity. Ninety-seven percent of the colonies were positive for deformed wing virus (DWV), 29% were positive for acute bee paralysis virus (ABPV) and 1.4% were positive for both sacbrood virus (SBV) and black queen cell virus (BQCV). Multiple infections were common, with 32% of the colonies were infected by more than one virus.

Keywords: Honeybee; *Apis mellifera*; RT-PCR; RNA viruses; Genetic diversity

1. Introduction

Viruses infecting honeybees have been isolated and characterised over the past 50 years, with there being over 18 known viruses described in the literature (Allen and Ball, 1996). The picorna-like single-stranded positive sense RNA viruses dominate this group and are consequently, the best studied to date. Honeybee viruses of this group include the deformed wing virus (DWV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), sacbrood virus (SBV), black queen cell virus (BQCV) and the Kashmir bee virus (KBV).

Single-stranded RNA viruses of the honeybee have been detected on a global scale, being present in the USA (Chen et al., 2004a), South America (Antunez et al., 2005), South Africa (Benjeddou et al., 2002), Australia (Anderson and Gibbs, 1988), Asia (Christian et al., 2005) and Europe (Bakonyi et al., 2002; Tentcheva et al., 2004). They are commonly described to cause inapparent, symptomless infections in their hosts and as a consequence, often go undetected (Bailey, 1967). Certain triggers, such as immune suppression of the honeybee caused by parasites, such as by the mite *Varroa destructor*, are considered to induce viral replication with viral symptoms consequently being observed (Yue and Genersch, 2005 Ball, 1993). Symptoms of virus infection include crumpled, deformed wings seen in bees infected with DWV; trembling, flightless bees seen in bees infected with CBPV and the accumulation of fluid in brood cells seen in bees infected with SBV (Bailey and Ball, 1991). As well as suppressing honeybee immunity, *V. destructor* is also considered important in vectoring viruses, passing them between honeybees (Bowen-Walker et al., 1999).

The wider implications of virus infection and *V. destructor* infestation are thought to be a reduction in honeybee populations, a decline in pollination and a reduction in honey production. They are therefore imperative to study and understand with respect to ecological processes as well as for commercial beekeeping practices.

In this study, we set out to analyse apiaries in Devon for the presence of 6 different viruses. Reverse transcription-PCR (RT-PCR) was employed to amplify regions of the

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0022-2011/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2008.02.010

replicase polyprotein of these viruses. The colonies which were positive for virus infection were sequenced and used to investigate the virus genetic diversity.

2. Materials and methods

Sixty honeybees were collected from 23 different apiaries in Devon, South West England. Samples were collected during September and October 2006, with the 60 bees comprising of 20 worker bees from three different healthy colonies within each apiary. Honeybees were incubated at −80 °C until processing when they were ground-up in liquid nitrogen to a fine powder. 0.03 g of this material was use to extract total RNA using the Qiagen RNeasy Mini Kit (Qiagen) following the manufacturer's instructions.

RNA was screened for the presence of DWV, CBPV, SBV, ABPV, BQCV and KBV using virus specific primers previously described by Tentcheva et al. (2004). RT-PCR was performed using the Qiagen OneStep RT-PCR kit (Qiagen) in 25 μl reactions containing 200 ng RNA template and 2.5 pmol each primer. Thermal reactions proceeded with an initial reverse transcription incubation at 50 °C for 30 min then incubation at 95 °C for 15 min. This was followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min. A final extension step was performed at 72 °C for 10 min. PCR products were electrophoresed through a 2% agarose gel containing 0.5 μg/ml ethidium bromide and subsequent visualisation by UV transillumination. PCR products were excised from the gel and extracted from the agarose using the Qiaex II gel extraction kit (Qiagen), following the manufacturer's instructions and was used as the template for sequencing reactions.

Sequencing reactions were carried out using the ABI Big-dye version 3.1 sequencing kit, following the manufacturer's instructions and were run on the ABI Prism 3100 Genetic analyser (Applied Biosystems). Sequence data was automatically collated and analysed using the ABI sequencing analysis software and was subsequently manually verified.

Similarities between virus sequences and published sequences were determined using BLAST (Basic Local Alignment Search Tool) within the National Centre for Biotechnology Information (NCBI) database (http://www.//ncbi.nlm.nih.gov/BLAST). Virus sequence data generated were deposited in GenBank, under Accession Nos. DQ434893-DQ434992.

3. Results and discussion

In this study we demonstrate the successful use of RT-PCR to detect honeybee viruses in apiaries in Devon, South West England. The apiaries were screened for six different viruses, with DWV, ABPV, SBV and BQCV being detected in the colonies. All these colonies had a history of infestation with the parasitic mite *V. destructor* at varying levels yet were considered to be relatively healthy, showing no abnormal symptoms at the time of sampling.

DWV was the most prevalent, with 97% of colonies screened being positive for the presence of this virus. This high occurrence of DWV has been seen in several other countries, including France, where Tentcheva et al. (2004) found more than 90% of the apiaries they studied to have DWV consistently throughout the course of an annual cycle. The two colonies which were found not to have DWV were considered to have no obvious differences to any of the other colonies both with respect to geographical location and also history of disease. It is therefore intriguing as to why these colonies are free from this virus at this time point and may be due to limits of the assay detection or due to the virus being more prevalent in these colonies at other times of the year.

Sequence analysis of the DWVs present in Devon showed a high genetic diversity, with nucleotide changes detected across the whole of the sequence amplified (Fig. 1). Forty-seven nucleotide changes were detected consistently across the sequence amplified with no regions obviously more conserved. Eighty-one percent of these substitutions occur at the 3rd position, with 11% and 8% occurring at the 1st and 2nd position, respectively. Despite the high nucleotide diversity, there is a high level of amino acid conservation, being identical in all isolates (including French and USA isolates) with the exception of five of the Devon infected colonies. This suggests a strong selection and high conservation amongst DWVs across the world.

Acute bee paralysis virus was the 2nd most abundant virus in Devon, being found in 29% of the colonies sampled. Less nucleotide substitutions were evident within the ABPV sequences amplified from Devon, compared to DWV. A higher sequence similarity was detected amongst the isolates detected in Devon, compared with other sequences in GenBank (Fig. 1). The ABPV isolates from Devon, showed high amino acid sequence similarities with other ABPVs from Hungary, Poland and South Africa, exhibiting identities of greater than 99.98%. This again, suggests a strong selection for sequence conservation amongst the ABPV group.

SBV was only detected in one of the colonies sampled in Devon. This contrasts with results of Tentcheva et al. (2004) which found 67% of worker bees in French apiaries across various regions to be infected with SBV in autumn. It may become apparent that this virus is more common in the summer months, a trend which was described by Tentcheva et al. (2004) being attributed to a difference in honeybee vulnerability to infection at different times of the year or to changes in the environment. Seasonal variations in viral abundance have additionally been identified in another single-stranded RNA virus, the *Solenopsis invicta* virus-1 (SINV-1), infectious to the red imported fire ant (Valles et al., 2007). Similarly, highest viral abundances were detected during the summer months, being linked to increases in the ant colony growth rate during this period.

A 5'(C/T_{97})T(G/A_{97})GATGG(G_{99}/A)**T(T_{99}/C)(C/T_{99})**GATTC(A/G_{91})ATATCTTGGAA(C/T_{97})ACTAGTGCTGGTTTTCC(A/T_{99})TTGTCTTCATTAAAGCCACC(C/T_{97})GGAACATC(G/A_{96})GGTAAGCGATGGTTGTT(C/T_{96})GA(C_{88}/T)ATTGAG(T/C_{97})TA**C(C/T/A$_{97}$)A**GA(C_{99}/T)TCGGGATGTTA(C/T_{96})CT(C_{96}/T)(C/T_{73})TGCG(C/T_{94})GGAATGCGTCC(C_{96}/T3)GA(G/A_{96})CTTGAGATTCAATTATC(A_{97}/G)AC(A/G_{96})AC(A_{96}/C/G)CA(A/G_{95})TTAATGAGG**AA(T/A$_{99}$)A(A_{99}/G)(A/G_{97})**GGAATAAAACCTCA(C_{97}/T)AC(A/T_{97})ATATT(C_{99}/T)ACGGATTGT(C/T_{95})TGAA(A_{99}/G*)GA(C/T_{99})ACTTGTTTGCCTGT(G_{51}/T)GAAAA(G/A_{97})TGT**A(A/G$_{99}$)A**ATACCTGGTAAGACTAG(A_{96}/G)ATATTTAGTATAAG(C/T_{97})CCGGTACA(A/G_{99})TTTAC(A/C_{52}/T_{48})ATACC(A/G_{92})TT(C/T_{96})(A/C$_{56}$)G(G/A_{96})CA(A/G_{97})TATTA(C_{88}/T)TTAG-3'

B 5'GAT**(A$_{96}$/C)CA**TT(A_{96}/G*)AAGGATGAGAGAAGACCAATTGAAAAAGTAGATCAACT(A_{88}/G*)AAAACACGTGTGTT(T_{96}/C*)**T(C$_{96}$/T)(T$_{83}$/C)**AATGGGCCTATGGACTTTTCTAT(T_{92}/C*)ACTTTTAGAATGTACTA(C_{88}/T*)TT(A_{96}/G*)GGCTT(T_{96}/C*)ATAGCACA(C_{96}/T*)CTTATGGA(G_{88}/A*)AATCG(A_{92}/G*)ATAACCAATGAAGT(G_{88}/A*)TCCATAGGAACTAATGTTTATTCCCAAGATTGG**(C$_{88}$/A*)AT**AAGACAGTTAGAAAACTTAAAACTATGGGACCCAAGGTTAT(C_{88}/T*)GCAGGAGATTT(C_{96}/T*)TCAACCTTTGA(T_{96}/C*)GGATCTTT(A_{96}/G*)AATGTTTGCATTATGGAAAAATT(T_{96}/C*)GC(C_{88}/T*)GAC(C_{92}/T*)TAGCGAA(C_{88}/T*)GAATTTTATGATGA(C_{92}/T*)GGA(C_{79}/T*)C(A_{92}/G*)GA(A_{96}/G*)AA(T_{92}/C*)GCA(T_{92}/C*)TAAT(T_{92}/A*)CGACATGT(C_{88}/T*)TTGCT(T_{96}/A)ATG**(G$_{96}$/A)AT**GTATA(C_{88}/T*)AA(C_{92}/T*)TC(A_{96}/T*)ACACA(T_{96}/C*)(**A$_{96}$/G*)TT**TGTGGTGATTC(C_{92}/T)GTATATATGATGACACA(C_{96}/T*)AG(T_{92}/C*)CA-3'

Fig. 1. Consensus nucleotide sequence for the DWV PCR products (corresponding to base positions 8592–8925 of the DWV genome, Accession No. AY292384) (A) and the ABPV PCR products (corresponding to base positions 5294–5712 of the ABPV genome, Accession No. AF150629) (B). The brackets (N/N) indicate all variable nucleotides at the respective position and the number denotes the % frequency of the dominant nucleotide. Substitutions which occur in isolates not detected in Devon are marked with an asterisk and substitutions within a codon, that potentially give rise to amino acid changes are highlighted in bold.

It is therefore postulated that this may also be the case with SBV. A high amino acid sequence similarity was observed between the Devon SBV and the SBV isolates detected in France and China (data not shown).

BQCV was found in a single colony, out of 69 which were tested, again differing from results found in other countries. Tentcheva et al. (2004) found BQCV in 75% of apiaries during the autumn in France, with Chen et al. (2004a) finding occurrences of 66% in apiaries in USA. A sequence identity (>99.98%) was shared between all BQCVs analysed, including strains from South Africa and USA (data not shown).

CBPV and KBV were not detected in any of the colonies sampled in Devon. The transmission of these two viruses between honeybees has not been as strongly associated with parasitic vectors, so their routes of transmission between bees is relatively unknown. To date, CBPV has not been identified as having a clear pattern of occurrence and distribution; however, it is known to suddenly be induced, causing disease (Bailey and Ball, 1991). It would therefore be of interest to continuously monitor this virus to see if any obvious trends relating to environmental parameters and disease can be identified.

Multiple infections have previously been described in honeybee populations (Chen et al., 2004b, 2005) and 32% of the colonies sampled in Devon had more than one virus present, with one colony having a triple infection. The dynamics of the virus populations within these colonies, co-occurring together, would be of great interest to study to see how they potentially fluctuate with respect to each other.

In summary, this report has provided evidence for four different honeybee viruses in Devon, UK, with the occurrence of multiple infections also being common. In general, the diversity of viruses (based on nucleotide sequences) in Devon is high, in particular, DWV. It is proposed that to acquire a better understanding of the occurrence and diversity of honeybee viruses in the UK a longer temporal study, encompassing different types of bees also, is required which will enable further conclusions to be made regarding viral transmission, induction and seasonal changes in virus occurrence.

Acknowledgments

We thank the C.B. Dennis Beekeepers Research Trust for funding of this research and the members of the Devon Beekeepers Association (R. Aitken, R. Ball, G. Berrington, B. Brassey, G. Davies, D. Dixon, B. Gant, J. Grist, A. Hawtin, J. Hewson, A. Hodgson, W. Holman, D. Milford, H. Morris, A. Normand, J. Phillips, D. Pratley, J. Richard-

son-Brown, F. Russell, R. Saffery, K. Thomas, C. Turner, A. Vevers, P. West) for their invaluable assistance in collecting the bees. DCS is a Marine Biological Association of the UK (MBA) Research Fellow funded by grant in aid from the Natural Environmental Research Council of the United Kingdom (NERC).

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