

Review

Development of molecular tools for honeybee virus research: the South African contribution

Sean Davison*, Neil Leat and Mongi Benjeddou

Department of Biotechnology, University of the Western Cape, Bellville 7535, South Africa.

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Increasing knowledge of the association of honeybee viruses with other honeybee parasites, primarily the ectoparasitic mite *Varroa destructor*, and their implication in the mass mortality of honeybee colonies has resulted in increasing awareness and interest in honeybee viruses. In addition the identification, monitoring and prevention of spread of bee viruses is of considerable importance, particularly when considering the lack of information on the natural incidence of virus infections in honeybee populations worldwide. A total of eighteen honeybee viruses have been identified and physically characterized. Most of them have physical features resembling picornaviruses, and are referred to as picorna-like viruses. The complete genome sequences of four picorna-like honeybee viruses, namely Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Sacbrood Virus (SBV) and Deformed Wing Virus (DWV) have been determined. The availability of this sequence data has led to great advances in the studies on honeybee viruses. In particular, the development of a reverse genetics system for BQCV, will open new opportunities for studies directed at understanding the molecular biology, persistence, pathogenesis, and interaction of these bee viruses with other parasites. This review focuses on the contribution of the Honeybee Virus Research Group (HBVRG), from the University of the Western Cape of South Africa, in the development of molecular tools for the study of molecular biology and pathology of these viruses.

Key words: Honeybee, virus, *Varroa destructor*, picorna-like, reverse genetics, RT-PCR, infectious clone, infectious RNA.

INTRODUCTION

The European honeybee (*Apis mellifera* L.) is found in all parts of the world, except for the extreme Polar Regions (Dietz, 1992). The wide distribution range of this species necessitated that it adapts to a broad range of climates, from cold temperate to tropical conditions and from areas with high rainfall to semi-deserts. Each region has its distinctive floral season, its complement of natural enemies, and its characteristic nesting sites (Eardley et al., 2001). Managed honeybee colonies are the main

source of pollinators of cultivated plants in tropical and temperate countries, because of their social structure, their behavior, and their anatomy (Johannesmeier and Mostert, 2001). The importance of honeybees to both agriculture and conservation exceeds the direct market value of honeybee products derived by beekeepers in terms of honey, bee-collected pollen, royal jelly, wax, venom, and health supplements. Honeybees are considered to be responsible for 60- 70 % of all pollination in South Africa (Johannesmeier and Mostert, 2001).

There has been an increasing interest in honeybee viruses due to their observed association with two species of honeybee parasitic mites, the tracheal mite (*Acarapis woodi*) and the varroa mite (*Varroa destructor*).

*Corresponding author. Fax: +21-21-9593505. Phone: +27 21 9592216. Email: SDavison@uwc.ac.za.

Varroa is of a particular concern as it has decimated honeybee colonies in many parts of the world. In its last continent of conquest, North America, it has been responsible for large-scale colony losses (Kraus and Page Jr., 1995; Finley et al., 1996). Even with extensive use of acaricides, reports from areas of the USA indicated more than 50% commercial colony losses and up to 85% loss of wild honeybee colonies (Finley et al., 1996; Kraus and Page Jr., 1995). However, there are strong suggestions that losses recorded in colonies infested with the mite are a result of an association between varroa and honeybee viruses rather than the mite acting alone (Bailey et al., 1983; Ball and Allen, 1988; Allen and Ball, 1996; Brødsgaard et al., 2000). This has led to the use of the term “bee parasitic mite syndrome” to describe such a disease complex.

BEE PARASITIC MITE SYNDROME

The varroa mite, *Varroa destructor*, is currently considered the major pest of honeybees in most parts of the world. It was first described as an ectoparasitic mite of the Asian honeybee, *Apis cerana*, from Indonesia in 1904. This bee has probably coevolved with the parasite, which adapted to keep the mite under control (Sammataro et al., 2000). The mite only infested *Apis mellifera* colonies when these were taken into areas where *A. cerana* were present. Movement of *Apis mellifera* by humans then ensured that the mite spread throughout the world (Swart et al., 2001).

Varroa infestations have proved difficult to control and impossible to eradicate. This is especially the case where there is a massive wild population of bees beyond reach, as is the case in South Africa (Allsopp, 1997; Swart et al., 2001). In Europe and the USA many hundreds of thousands of commercial honeybee colonies have died as a result of varroaosis (pathology caused by varroa), and beekeeping is no longer possible without some anti-varroa treatment (Allsopp, 1997). The collapse of colonies however, is not solely attributed to the effect of varroa. Colonies with large numbers of varroa mites are weakened further by other diseases and pests, including viruses (Swart et al., 2001). These secondary infections may have caused the total collapse of the colonies.

The term “bee parasitic mite syndrome” has been used to describe a disease complex in which colonies are simultaneously infested with mites and infected with viruses and accompanied with high mortality (Shimanuki et al., 1994). The relationship between mite infestation and virus infection is not clearly understood. Although the mite has been demonstrated to act as an activator of inapparent virus infections and as a virus-transmitting vector (Ball and Allen, 1988; Bowen-Walker et al., 1999), no direct link between the actual mite population and colony collapse has been found (Martin, 1998).

In South Africa, the Black Queen Cell Virus, the Acute Bee Paralysis Virus and another two unidentified viruses have been shown to be implicated in increased honeybee mortality in African honeybee colonies infested with varroa mite (Swart et al., 2001). Large numbers of opened cells with dead, pink-eyed pupae were seen in honeybee colonies with high varroa loads. These pupae had virus levels many times higher than those of healthy pupae removed from the same colonies, suggesting that viruses were the cause of this mortality (Swart et al., 2001).

HONEYBEE VIRUSES

A total of eighteen honeybee viruses have been identified and physically characterized (Allen and Ball, 1996). In addition, the complete genome sequences of four honeybee viruses, namely ABPV (Govan et al., 2000), BQCV (Leat et al., 2000), SBV (Ghosh et al., 1999) and DWV (Lanzio and Rossi, unpublished), have been determined. Most honeybee viruses resemble picornaviruses, but there is currently not sufficient information for classification. Phylogenetic studies on the ABPV, BQCV, SBV and DWV revealed that these viruses are not members of the picornavirus family. SBV, and probably DWV, appears to be most closely related to the infectious flacherie virus of silkworms (Ghosh et al., 1999). BQCV and ABPV belong to a novel group of insect infecting RNA viruses, the *Dicistroviridae* family, which has recently been described (Govan et al., 2000; Leat et al., 2000).

The *Dicistroviridae* family includes Cricket paralysis virus (CrPV), *Drosophila C* virus (DCV), *Plautia stali* intestine virus (PSIV), *Rhopalosiphum padi* virus (RhPV), and Himetobi P virus (HiPV) (van Regenmortel et al., 2000). The genomes of CrPV, DCV, PSIV, RhPV and HiPV are monopartite and bicistronic with replicase proteins encoded by a 5'-proximal ORF and capsid proteins by a 3'-proximal ORF. In the case of PSIV, translation initiation of the 3'-proximal ORF has been demonstrated to be dependent on an internal ribosome entry site (IRES), starting at a CUU codon (Sasaki and Nakashima, 1999). Similarly, it has been suggested that translation initiation of the 3'-proximal ORF of BQCV is facilitated by an IRES at a CCU codon (Leat et al., 2000).

Most honeybee viruses can remain in a latent state within individuals and may spread within bee populations at this low level of inapparent infection (Allen and Ball, 1996; Ball, 1997). SBV and chronic paralysis virus (CPV), however, can reliably be diagnosed by symptoms occurring naturally in honeybee colonies (Allen and Ball, 1996). When allowed to undertake their normal activities unhindered, bees may have developed an ability to resist the multiplication and spread of viral infections (Allen and Ball, 1996; Ball, 1997). However, under certain

circumstances virus replication is triggered and infection can spread between bees, leading to outbreaks of disease. These outbreaks of overt disease may be spectacular and lead to significant losses (Allen and Ball, 1996; Ball, 1997).

MOLECULAR CHARACTERIZATION OF BQCV

Despite their economic and ecological impact, comprehensive molecular studies on honeybee viruses have only recently been initiated. This is surprising given the fact that the majority of these viruses are remarkably amenable to molecular analysis. The HBVRG set about providing a foundation for molecular studies on honeybee viruses. For this purpose, the complete nucleotide sequence of the genomes of two important viruses identified as being associated with varroa mite infestations in South Africa, BQCV and ABPV, was determined (Govan et al., 2000; Leat et al., 2000). Later on, and because of its association with two of the most important and widespread parasites of the honeybee, BQCV was identified as an ideal candidate among honeybee viruses for an in-depth molecular study.

BQCV was originally found in dead honeybee queen larvae and pupae (Bailey and Woods, 1977) and has been shown to be the most common cause of death of queen larvae in Australia (Anderson, 1993). The virus has isometric particles 30 nm in diameter and has a single-stranded RNA genome of 8550 nucleotides excluding the poly(A) tail (Leat et al., 2000). In addition to its association with *Varroa destructor* in South Africa and in many parts of the world, BQCV is known for its universal association with the microsporidian parasite *Nosema apis* (Bailey et al., 1983; Allen and Ball, 1996). *Nosema* disease is probably the most widespread disease of adult honeybees (Swart et al., 2001).

MOLECULAR IDENTIFICATION OF HONEYBEE VIRUSES

The identification of bee viruses is of considerable importance, particularly when considering the lack of information on the natural incidence of virus infections in honeybee populations worldwide. However, because virus particles are often present in small numbers and can only be detected by laborious infection experiments, viruses are not yet included in the health certification procedures for honeybee imports and exports (Allen and Ball, 1996). Developing a sensitive diagnostic technique would help to identify viruses present in bees under natural conditions, and could be used to screen virus preparations, employed in research, to ensure they are free of other contaminant viruses.

Several methods have been used to detect honeybee

viruses including immunodiffusion, enzyme-linked immunosorbent assay (ELISA), enhanced chemiluminescent western blotting and RT-PCR (Allen and Ball, 1996; Allen et al., 1986; Stoltz et al., 1995). The most commonly used among these methods is still the immunodiffusion test because it is rapid, inexpensive and specific (Allen and Ball, 1996). However, the serological methods have the drawbacks of limited availability of antisera and questions regarding the specificity of some antisera as a result of antiserum production from preparations containing virus mixtures. Raising antisera is also a time consuming process with a large amount of a given virus needed to raise the antiserum. Consequently, the use of the serological method would be limited to laboratories that can produce large amounts of pure virus to raise a library of suitable antisera. By contrast, a diagnostic technique using RT-PCR can be rapidly implemented in independent laboratories after the basic protocol and primer sequences are made available.

RT-PCR has been used to detect a variety of RNA viruses including the picorna-like insect viruses (Chungue et al., 1993; van der Wilk et al., 1994; Stoltz et al., 1995; Canning et al., 1996; Stevens et al., 1997; Harris et al., 1998; Johnson and Christian, 1999; Hung and Shimanuki, 1999). The technique is reliable, specific and sensitive. However, RT-PCR experiments on insects are usually hampered by the problem of inhibitory components, which compromise reverse transcription and PCR reactions (Chungue et al., 1993). To overcome this problem, many RNA extraction methods have been developed or modified in order to remove these inhibitors (Singh, 1998).

The availability of virus sequence data raised the possibility of using the RT-PCR technique for the identification and detection of BQCV and ABPV. Unique PCR primers were designed against BQCV and ABPV and a basic protocol was developed for the detection of these two viruses using a reverse transcriptase PCR (RT-PCR) assay (Benjeddou et al., 2001). To overcome the problem of inhibitory substances for RT-PCR, total RNA was extracted from infected and healthy bee pupae using the Nucleospin RNAII total RNA isolation Kit of Macherey-Nagel. The kit uses the combined guanidinium thiocyanate and silica membrane methods. This method has been successfully used in aphids, plants and mosquitoes (Stevens et al., 1997; Naidu et al., 1998; Chungue et al., 1993; Harris et al., 1998). Primers were tested against purified virus particles and virus-infected bees, and sensitivities were of the order of 130 genome equivalents of purified BQCV and 1600 genome equivalents of ABPV (Benjeddou et al., 2001).

The RT-PCR assay developed in this study could become a standard method in the health certification for honeybee (and honeybee products) imports and exports, and in the screening of virus preparations used in research. However, it is necessary to improve this system

in terms of sensitivity and the number of viruses included. This assay must be tested against field samples, to find out whether it will help to identify viruses present in bees under natural conditions. It is also important to continue the genome sequencing of other honeybee viruses, to enable the development of similar assays for these viruses, and finally to develop a multiplex PCR system in which multiple viruses could be identified simultaneously using a single reaction.

REVERSE GENETICS TECHNOLOGY

The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into their genomes, a technique known as reverse genetics (Pekosz et al., 1999). Such reverse genetics systems have been developed for a number of positive-stranded RNA viruses, including *Picornaviruses*, *Caliciviruses*, *Alphaviruses*, *Flaviviruses*, and *Arteriviruses*, whose RNA genomes range in size from ~7 to 15 kb in length (Yount et al., 2000). The production of cDNA clones and/or PCR-amplicons, from which infectious RNA can be transcribed *in vitro*, is an essential step in the development of reverse genetics systems for these viruses. The availability of these clones/PCR-amplicons has facilitated the study of the genetic expression and replication of RNA viruses by use of mutagenesis, deletions, and insertions and by complementation experiments. It has also enhanced the understanding of the molecular mechanisms of natural or induced RNA recombination, and of plant-virus interactions such as cell-to-cell movement. This has resulted in the development of new viral vectors and vaccines (Boyer and Haenni, 1994).

The development of the reverse genetics technology for honeybee viruses will facilitate the creation of chimeric viruses or the introduction of specific mutations into their viral genomes. The first step in the development of this technology for BQCV or any other honeybee virus must be the demonstration of the infectivity of its naked RNA. Since there is no cell culture system available for honeybee viruses, it is necessary to demonstrate the ability of naked genomic RNA from BQCV to initiate an infection upon direct injection into live bee pupae.

INFECTIVITY OF NAKED HONEYBEE VIRAL RNA

BQCV viral RNA was tested for infectivity in honeybee pupae (Benjeddou et al., 2002a). Total viral RNA was extracted from previously infected bee larvae and then quantified with an UV spectrophotometer. Several preparations of viral RNA were made in order to examine its infectivity. The preparations were as follows: a positive control of purified BQCV particles, BQCV particles

treated with RNase A, viral RNA, and viral RNA treated with RNase A. Phosphate buffer was used as a negative control. Two microlitres of each preparation was injected into bee pupae. The bee pupae were incubated at 30-35°C for 8 days prior to the virus being purified. The formation of BQCV virus particles generated by injection of bee pupae with naked genomic RNA were readily observed by electron microscopy. In a parallel control, virus particles were also generated from injection with pure BQCV particles and BQCV particles treated with RNase A, but not from injection with RNase A-treated viral RNA or with phosphate buffer. This clearly showed that RNase A abolishes the infectivity of naked RNA while having no influence on the infectivity of intact particles

Demonstrating that BQCV naked RNA is infectious in bee pupae made it possible to overcome the limitation of not having a cell culture system, and to design experiments aimed at developing a system for the production of infectious clones/transcripts for this virus.

DEVELOPMENT OF INFECTIOUS TRANSCRIPTS FOR BQCV

The production of infectious RNA transcripts from PCR-amplicons has become a method of choice for many investigators because of the improvements in the PCR in terms of fidelity and length of amplification. The pioneering work of Gritsun and Gould (1995 and 1998) has also resulted in the improvement of this method by using a combination of primer sets and optimising their concentrations. This method is simple, rapid, and it overcomes the problem of instability of certain sequences in bacteria (Hayes and Buck, 1990; Gritsun and Gould, 1995; Tellier et al., 1996; Gritsun and Gould, 1998; Campbell and Pletnev, 2000).

Benjeddou and co-workers from the HBVRG (2002b) have described the manipulation of the BQCV genome using long reverse transcription-PCR, and adapting systems and strategies used in previous studies (Gritsun and Gould, 1995; Tellier et al., 1996; Gritsun and Gould, 1998). A genetic marker mutation was introduced into BQCV by PCR-directed mutagenesis. The fusion-PCR method, used for mutagenesis, was a combination of the method used by Gritsun and Gould (1995) with that of Rebel *et al.* (2000). The mutation abolished one of two *EcoRI* sites, 182 bases apart. This genetic marker mutation was used to clearly demonstrate that viral particles, recovered from these experiments, originated from infectious transcripts, and were not simply the product of an activated inapparent infection.

In this study, primers were designed for the amplification of the complete genome, the *in vitro* transcription of infectious RNA, and PCR-directed mutagenesis. An 18-mer antisense primer was designed for reverse

transcription (RT) to produce full-length single-stranded cDNA (ss cDNA). Unpurified ss cDNA from the RT reaction mixture was used directly as a template to amplify the full genome using long high fidelity PCR. The SP6 promoter sequence was introduced into the sense primer to transcribe RNA directly from the amplicon. RNA was transcribed *in vitro* with and without the presence of a cap analog and injected directly into bee pupae and incubated at 30-35°C for 8 days. *In vitro* transcripts were infectious but the presence of a cap analog did not increase the amount of virus recovered. A single base mutation abolishing an *EcoRI* restriction site was introduced by fusion-PCR, to distinguish viral particles recovered from infectious transcripts from the wild type virus (wtBQCV). The mutant virus (mutBQCV) and wtBQCV were indistinguishable using electron microscopy and western blot analysis. The *EcoRI* restriction site was present in wtBQCV and not in mutBQCV (Benjeddou et al., 2002b).

The development of this reverse genetics system for BQCV was the first for honeybee viruses. The development of this technology will facilitate the creation of chimeric viruses, or the introduction of specific mutations into their viral genomes. Such mutant viruses produced have the potential to be used in new experiments aimed at understanding the bee parasitic mite syndrome.

FUTURE POSSIBILITIES

The development of a reverse genetics system for BQCV, and especially the fact that its genome can be manipulated, has opened new opportunities for studies aimed at exploring the potential of this virus as a vector to express foreign proteins. The insertion of a sequence encoding the green fluorescent protein (GFP) is of a particular interest, because of the significant advantages that this gene offers over other marker genes such as the *Escherichia coli UidA* gene (GUS) (Baulcombe et al., 1995). Among many other advantages, the fluorescence of GFP is an intrinsic property and does not depend on exogenous substrates or co-factors, the simplicity and ease of detecting the fluorescence, and more importantly the GFP is non-toxic and appears not to interfere with cell growth and function (Baulcombe et al., 1995). In addition, the production of new virus recombinants carrying the GFP-encoding sequence will improve the previously described marker-mutation virus system. BQCV mutants (mutBQCV) produced in the previous system carried a marker mutation, and were proposed for use in new experiments aimed at understanding the bee parasitic mite syndrome. The large size of the GFP -encoding sequence (717 bp) will also offer a challenge regarding the BQCV's uptake capacity for foreign sequences.

Efforts should also be made to construct BQCV virus-based vectors to express other proteins, including antigens of viruses, bacteria and parasites of the honeybee. In the future, success may be achieved in making single constructs that will express multiple foreign proteins for use against multiple honeybee disease agents.

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