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Full Length Research Paper

Detection of three honeybee viruses simultaneously by a single Multiplex Reverse Transcriptase PCR

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A single multiplex reverse transcriptase (RT) polymerase chain reaction (PCR) assay was developed for the simultaneous detection of three honeybee viruses: acute bee paralysis virus (ABPV), sacbrood virus (SBV) and black queen cell virus (BQCV). Unique PCR primers were designed from the complete genome sequence to amplify fragments of 900 bp from ABPV, 434 bp from SBV and 316 bp from BQCV. Individual bee pupae homogenates or total RNA extracted from these crude extracts were used in the RT-PCR amplification. Sequence analysis of the fragments amplified revealed nucleotide sequence identities between 97 and 98% for each virus against its reference strain. In a blind test, samples containing various combinations of ABPV, SBV and BQCV were successfully identified. Furthermore, field samples of honeybee pupae were screened for viral infections, and evidence of virus inapparent infection as well as virus co-infection were found.

Key words: Multiplex, RT-PCR, virus, honeybee, varroa.

INTRODUCTION

Viral diseases of honeybees have become increasingly important to the honeybee keepers and all related agricultural industries. These viruses are distributed worldwide, and together with pollution and the use of insecticides the resulting high honeybee mortality rates pose a real threat to these industries (Morse and Calderone, 2000; Spira, 2001). To date 18 different honeybee viruses have been identified and characterised and most of them persist as inapparent infections, which cause sub-lethal infections in apparently healthy bees (Allen and Ball. 1996; Bailey et al., 1983; Ball and Allen, 1988). Inapparent infections make viral diagnosis difficult due to the lack of gross symptoms shown (Bailey, 1965). The parasitic mite *Varroa destructor* (formerly named *Varroa jacobsoni*) has been suggested to play a pivotal role in honeybee mortality (Allen and Ball. 1996; Bailey et al., 1983; Ball and Allen, 1988; Brødsgaard et al., 2000). Yet the relationship between the mite infestations and virus infections is not clearly understood although the mite has been demonstrated to act as a vector (Ball and Allen, 1988; Bowen-Walker et al., 1999) or activator (Ball and Allen, 1988; Brødsgaard et al., 2000) of the inapparent viruses found in bees. The genomes of the viruses included in the present study have been completely sequenced, namely acute bee paralysis virus (ABPV), sacbrood virus (SBV) and black queen cell virus (BQCV) (Govan et al., 2000; Ghosh et al., 1999; Leat et al., 2000). All of these viruses have been referred to as picorna-like viruses due to their physical features, although ABPV and BQCV have been seen to differ from mammalian picornaviruses (Govan et al., 2000; Leat et al., 2000). Each of the three viruses has 30 nm particles, and they are morphologically indistinguishable from each other (Allen and Ball, 1996). ABPV was first discovered as an inapparent infection during laboratory experiments (Bailey et al., 1963). This paralysis virus is spread by way of salivary gland secretions of adult bees, which are infected and in turn contaminate brood food stores with these secretions (Ball, 1985). ABPV infections are known to be widespread if the colonies are infested with *Varroa destructor* (Allen and Ball, 1996; Ball and Allen, 1988).

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ABPV has a single positive strand RNA genome consisting of 9470 nucleotides excluding the poly (A) tail (Govan et al., 2000). BQCV was originally detected in dead queen larvae and pupae (Bailey and Woods, 1977) and is also very often detected when colonies are infested with the microsporidian mite *Nosema apis* (Allen and Ball, 1996; Bailey et al., 1983). *N. apis* may be implicated in the mortality of honeybees when infected with the parasite which infects the epithelium of the bees midgut and causes increased susceptibility of the alimentary tract to infection by BQCV (Bailey et al., 1983). BQCV has a single positive strand RNA genome consisting of 8550 nucleotides excluding the poly (A) tail (Leat et al., 2000). SBV was first described in 1917 but later isolated by Bailey et al. (1964). The virus was so called due to the sac like appearance of the diseased larvae (Allen and Ball, 1996). SBV mainly infects honeybee larvae causing very discernable symptoms (Bailey, 1969), but can also multiply in young adult bees without causing any symptoms, this enables the virus to persist in colonies from one year to another (Bailey and Fernando, 1972). Usually SBV is not detected in hives because the worker bees remove diseased larvae as soon as possible (Bailey and Woods, 1977). SBV has a single positive strand RNA genome consisting of 8832 nucleotides (Ghosh et al., 1999).

Methods used to detect honeybee viruses include immunodiffusion techniques (Allen and Ball, 1996), enzyme linked immunosorbent assay, enhanced chemiluminescent western blotting (Allen et al., 1986) and reverse transcriptase (RT) PCR (Benjeddou et al., 2001; Grabensteiner et al., 2000; Stoltz et al., 1995). The most common technique still used is the immunodiffusion test because it is rapid, relatively inexpensive and specific (Allen and Ball, 1996). However serological methods employed have negative aspects when antisera is raised to samples that contain virus mixtures. Evans (2001) found evidence that ABPV and kashmir bee virus (KBV) can coinfect the same bee at one time. Therefore when inapparent viruses are present in the preparation of the virus of interest, antisera is raised to all the viruses present in that sample. Serological methods are also limited to laboratories that can produce large amounts of pure virus and then raise the suitable antisera to those viruses. RT-PCR, however, has shown to be a simple and rapid technique for detecting viruses. A variety of RNA viruses have been detected by RT-PCR including, rhinoviruses (Steininger et al., 2000), human herpes virus 6 (Norton et al., 1999.) and viruses found in plants and aphids (Singh, 1998). RT PCR has also been developed for honeybee viruses such as KBV (Evans, 2001; Hung and Shimanuki, 1999), SBV (Grabensteiner et al., 2000), BQCV (Benjeddou et al., 2001) and ABPV (Benjeddou et al., 2001, Evans, 2001). An advantage of RT-PCR detection of honeybee viruses is the genetic comparison and classification of different virus strains that could be rapidly carried out by sequencing the PCR products (Grabensteiner et al., 2000). Multiplex RT-PCR (M-RT-PCR) for the simultaneous detection of different viruses has been proposed for numerous viruses. These include fish viruses (Williams et al., 1999), Parainfluenza viruses (Aguilar et al., 1999; Echevarría et al., 1998.), viruses of olive trees (Bertolini et al., 2001), viruses found in the environment (Tsai et al., 1994) and food samples (Rosenfield and Jaykus, 1999), sugar beet viruses (Meunier et al., 2003) and potato viruses (Nie and Singh, 2000). M-RT- PCR reduces time and costs of reagents and has been proven to be specific and reliable (Singh et al., 2000). In order to overcome inhibitory components in the PCR there have been many RNA extraction methods developed or modified to remove these inhibitors (Singh, 1998).

The aim of this study was to develop an M-RT-PCR assay for the simultaneous detection of ABPV, SBV and BQCV.

MATERIALS AND METHODS Virus

propagation and purification

Apparently healthy bee pupae were collected from hives in the Stellenbosch area of the Western Cape, South Africa. The three viruses were propagated by injecting apparently healthy white to pink- eyed pupae with 2 µl of viral preparations. The pupae were then incubated for 6 to 7 days at 30°C. The viruses were purified as described by Leat and co-workers (2000).

RNA purification

Total RNA extraction was performed by adding 1 volume of 2% SDS (sodium dodecyl sulphate) (w/v) in 1X TE (Tris EDTA) to 100 µl extracted virus and heated at 65°C for 5 min. This was followed by phenol extraction and ethanol precipitation. Care was taken to ensure that phenol and ethanol was completely removed before continuing with the M-RT-PCR. The samples were stored at –70°C. RNA concentration was quantified by a UV spectrophotometer (Sambrook et al., 1989).

Bee crude extract

Homogenates of honeybee pupae were also prepared by homogenising individual bee pupae in 200 µl of 0.01 M phosphate buffer providing a crude extract. This homogenate was stored at –20°C for later use. The homogenates were centrifuged at 14000 rpm for one minute and an aliquot of the supernatant was heated at 90°C for 5 min before adding to the RT-PCR mix.

Oligonucleotide primers

The PCR primers were designed within a 1 kb region from the 3' end of each individual virus genome (Table 1). The primers were designed based on the complete genome sequence of ABPV (accession number AF150629), BQCV (SA) (accession number AF183905) and SBV (accession number AF092924) . ABPV primers were used previously in Benjeddou and co-workers (2001). Due to slight variations in the genome sequences of different strains of ABPV, SBV and BQCV deoxyinosine residues (denoted as I) were

Table 1. Multiplex primers for detection of three honeybee viruses.

Primer	Sequence	Amplicon size	Position
ABPVF*	5'TTATGTGTCCAGAGACTGTATCCA I 3'	900 bp	8460-8484
ABPVR*	5'GCTCCTATTGCTCGGTTTTTCGGT I 3'		9336-9360
BQCVF	5' GGAGATGTATGCGCTTTATCGAG I 3'	316 bp	7882-7904
BQCVR	5' CACCAACCGCATAATAGCGATTG I 3'		8176-8198
SBVF	5'GTGGCGCGCCCATTACTGTAGTG I 3'	434 bp	8169-8191
SBVR	5' CTCGACAATTCTCCCTAGTAGCC I 3'		8581-8603

*Primers used by Benjeddou et al. (2001).

incorporated at the 3' end of each primer. This ensured that the primer annealed to the template even if a strain specific mismatch occurred. Primer specificities were checked by using the basic local alignment tool (BLAST) (Altschul et al., 1990) from the National Centre for Biotechnology Information (NCBI) as well as being compared to all the honeybee virus sequences available in the database. The maximum degree of sequence identity between primers and non-corresponding genome sequences was 65% (data not shown). PCR conditions were optimised after extensive trials with primer ratios and annealing temperatures to enhance the amplification of each virus.

Reverse transcription and PCR amplification

Amplification was carried out in a total volume of 25 µl with the master mix containing 0.2 mM of each deoxynucleoside triphosphate. Both the extracted RNA and the bee homogenate extract were used in the Titan RT-PCR system (Roche Diagnostics Gmb Roche Biochemicals Manneheim Germany) following the manufacturers recommendations except as otherwise stated. The primer concentrations were as follows, ABPVR 0.08 µM and ABPVF 0.064 µM, SBVR 0.16 µM and SBVF 0.73 µM, BQCVR 0.24 µM and BQCVF 0.8 µM. The master mix also contained 4.8 mM DTT, 5 µl of 5X RT-PCR buffer, 1 µl of template RNA with 0.4 µl Titan polymerase mix. Reverse transcription and amplification took place with a continuous RT-PCR method in a GeneAmp Perkin Elmer 2400 thermocycler. The cycles stages were: RT 58°C for 30 min followed by denaturation at 94°C for 2 min. This was followed by 10 cycles of 94°C for 30 s, 63°C for 30 s and 68°C for 1 min. Then followed 25 cycles of 94°C for 30 s, 63°C for 30 s and 68°C for 1 min plus 5 s each cycle thereafter this was followed by a final extension of 68°C for 7 min. The PCR products were visualised on a 3% agarose gel containing ethidium bromide.

Nucleotide sequencing and analysis

Single bands were excised from the gel and purified using the High Pure PCR product purification kit (Roche). Purified products were then sequenced in both directions and analysed on an ABI Prism 3100 Genetic Analyser. The sequence analysis results of ABPV, SBV and BQCV amplicons were aligned with the published full-length sequences in the NCBI databases.

Blind tests and natural infections

Forty samples of laboratory- infected bees with various combinations of ABPV, SBV and BQCV were prepared and blindly tested; to investigate weather this method will correctly identify virus infections. A further 40 field samples of apparently healthy honeybee pupae were also screened for viral infections, using the method described herein. In both instances crude extracts of individual bees were used in the tests.

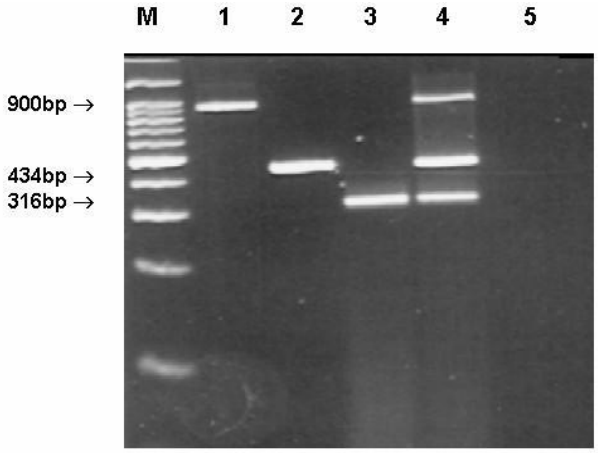

Figure 1. Amplification of ABPV, SBV and BQCV in honeybees individually and by M-RT-PCR. Extracted RNA from laboratory infected bee pupae and healthy bee pupae were used with the corresponding primers and combination of primers for M-RT-PCR. Lane M, 100 bp marker (Promega); lane 1, APBV-infected bee pupae; lane 2, SBV–infected bee pupae; lane 3, BQCV-infected bee pupae; lane 4, multiplex of ABPV plus SBV plus BQCV-infected bee pupae; lane 5, water (negative control).

RESULTS

Total RNA and extracted bee homogenate were used in the M-RT- PCR as described earlier. When using total RNA, ABPV, SBV and BQCV primers amplified each virus in uniplex at the predicted molecular weight of ABPV 900 bp, SBV 434 bp and BQCV 316 bp. Furthermore, the results of the sequencing and BLAST searches (Altschul et al., 1990) of the purified PCR products showed that they belong to the corresponding ABPV, SBV or BQCV, with 97 to 98% identities to the particular reference strains (data not shown). A cocktail of all primer pairs also

correctly amplified ABPV, SBV and BQCV as a multiplex. The primers failed to amplify any secondary viruses, which could have been present (Figure 1).

To further test the primers, total RNA and bee homogenates were used in a M-RT-PCR to detect ABPV, SBV and BQCV in various combinations (Figure 2 lanes 1 through 12). The positive controls were carried out with ABPV, SBV and BQCV RNA stocks and samples in lane 5 through 12 used extracted bee homogenates. The primer sets only amplified the desired product of the particular virus or viruses in each reaction.

A blind test was also carried out on 40 samples of bees infected with ABPV, SBV, BQCV and healthy bees. M-RT-PCR successfully identified the samples containing ABPV, SBV and BQCV in all instances. This assay was tested further against field samples, to find out whether it will help to identify viruses present in bees under natural conditions. Forty field samples of apparently healthy honeybee pupae were screened for viral infections, using the method described herein. Crude extracts of individual bee pupae randomly collected from the same colony were used in the RT-PCR tests. Thirty- four samples were negative, 5 samples tested positive for SBV, and one sample tested positive for both BQCV and SBV.

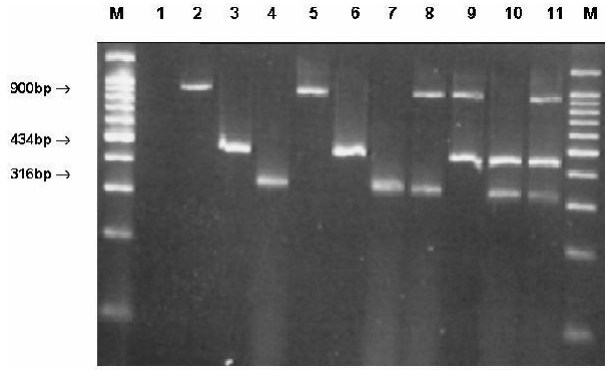

Figure 2. Detection of ABPV, SBV, and BQCV individually and as a M-RT-PCR. The specificity of the primers was tested on ABPV, SBV and BQCV virus stocks (positive controls) and laboratory infected pupae using a cocktail of primers in the M-RT-PCR. Lane M, 100 bp marker (Promega); lane 1 water (negative control); lane 2 ABPV virus stock (positive control); lane 3, SBV virus stock (positive control); lane 4 BQCV virus stock (positive control); lane 5 ABPV (infected pupae); lane 6 SBV (infected pupae); lane 7 BQCV (infected pupae); lane 8 ABPV plus BQCV (infected pupae); lane 9 ABPV plus SBV (infected pupae); lane 10 SBV plus BQCV (infected pupae); lane 11, ABPV plus SBV plus BQCV (infected pupae).

DISCUSSION

This report demonstrates the feasibility of M-RT-PCR using specific primers for the positive identification of several honeybee viruses in a single step reaction. The present assay saves costs and time in comparison to individual PCR assays which have been performed (Benjeddou et al., 2001; Evans, 2001; Grabensteiner et al., 2000; Hung and Shimanuki, 1999). It also has a potential as a tool in studying virus inapparent infections. In the absence of a standard method to quantify virus concentrations, due to the lack of a culture system for honeybees, the assay was optimised to a point where it can detect lower levels of virus in inapparently infected bees showing no signs of symptoms. It is noteworthy, however, that although field samples contained only apparently healthy honeybee pupae, they were collected from a colony that was previously infested with varroa to increase the chances to find virus infections. Crude extracts of individual bee pupae randomly collected from the same colony were used in the RT-PCR tests, and showed simultaneous and single infections as well as no infections. Thirty-four samples were negative, 5 samples tested positive for SBV, and one sample tested positive for both BQCV and SBV.

The simultaneous detection of BQCV and SBV is not unusual. Indeed, the sample from which BQCV was first isolated was a mixed preparation that also contained SBV (Bailey and Woods, 1977). This was thought to be due to the use of apparently healthy but SBV-infected bee pupae to propagate BQCV. Leat and co-workers (2000), however, argued that the simultaneous infection of these 2 viruses does exist, and that is due to their wide distribution rather than a specific relationship between them. Dall (1985) found no evidence of mixed infection in the case of Kashmir bee virus (KBV) and SBV. Working on BQCV, KBV and SBV inapparent infections, Anderson and Gibbs (1988) also found no pupae infected with more than one virus. However, they suggested that pupae were inapparently infected with more than one virus, but only one virus can be activated to detectable concentrations. The activated replicating virus would suppress the activation of the other two viruses (Anderson and Gibbs, 1988). In agreement with this argument, Evans (2001) presented evidence that ABPV and KBV can infect the same honeybee simultaneously, and further noted that KBV levels appeared lower than those of ABPV.

The assay presented here can further be optimised, and improved by adding more bee viruses as the genome sequences become available. It can be useful in studying virus inapparent infections, as well as in investigating suppression between viruses in case of simultaneous infections.

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