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## CHAPTER THREE

### Partial sequence of Kashmir bee virus (Indian strain) capsid coding region and interactions during PCR

#### 3.1 Abstract

Honeybee virus Kashmir bee virus (KBV) occurs worldwide with many different strains from these areas. A strain from India was used during the development of a multiplex reverse transcriptase (RT) PCR assay. The results which were obtained were inconsistent with regard to the amplification of virus templates that were expected. An attempt was made to determine the cause of the inconsistencies and also characterise the virus cDNA which was synthesised from the viral RNA. A 2 kilobase (kb) length of the capsid gene in the 3' region of this KBV India (KBV-in) strain was sequenced. The KBV-in strain was compared to the full length KBV reference strain sequenced in a database and also partial KBV sequences. The 2 kb sequence of KBV was also aligned with the full length ABPV sequence to determine the homology between these two viruses. Sequence attained from the KBV-in strain was compared to other KBV strains and was seen to be 72% homologous to the full and partial KBV strains over the specific region. The possible interaction of KBV to ABPV virus in a multiplex RT PCR showed that the primers designed for the RT-PCR were 87% homologous

to ABPV respectively. It was also concluded that the primer design for PCR assays detecting honeybee viruses is critical to prevent false positive amplification using this rapid screening method.



### 3.2 Introduction

The honeybee *Apis mellifera* L. is a very important insect that assists with the pollination of approximately 60-70% of flowering plants in South Africa (Johannesmeier & Mostert 2001). Honeybees are however, attacked by many different parasites and pathogens including viruses and bacteria. Kashmir bee virus (KBV) is thought to persist as an inapparent infection and was first thought to have been detected in *Apis cerana* colonies in Kashmir India (Bailey & Woods, 1977). However KBV has also been found in other parts of the world in *A. mellifera* and has made the initial finding of the virus difficult to prove (de Miranda *et al.*, 2004). Complete sequence analysis exists for a KBV strain from Pennsylvania USA which was seen to have 9524 nucleotides (de Miranda *et al.*, 2004); many other KBV strains from different parts of the world have partial sequences deposited into databases. A closely related virus both serologically and biologically to KBV is acute bee paralysis virus (ABPV) (Allen & Ball, 1995; Anderson, 1991). ABPV was first discovered during laboratory experiments on chronic paralysis virus and was found to be extremely virulent in adults and larvae when injected (Bailey *et al.*, 1963). The ABPV genome has been sequenced and was seen to have 9494 bp (Govan *et al.*, 2000). KBV and ABPV were said to be very closely related and it was even suggested that KBV was a strain of ABPV. However it was later found that these two viruses are not identical in the VP4 proteins and therefore suggested to be serologically distinct (Stoltz *et al.*, 1995). It was also found by capsid protein profiles and serology that KBV is more variable than ABPV (Bailey *et al.*, 1979; Allen & Ball, 1995).

Both viruses have also been detected in the same colony (Hung *et al.*, 1996) and also in the same bee (Evans, 2001); what this relationship is indicative of is still unknown.

Many of these honeybee viruses persist as inapparent infections that are activated by some or other stimuli. The *Varroa destructor* mites and virus infections have been associated with colony collapse (Ball, 1985; Allen *et al.*, 1986). The mites have also been suggested to act as vectors (Ball & Allen, 1988; Bowen Walker *et al.*, 1999) or stimuli for the virus to start multiplying (Ball & Allen, 1988; Brødsgaard *et al.*, 2000).

In the study completed in chapter two a multiplex RT PCR was designed to identify three viruses infecting honeybees. Initially KBV was included but due to primer interactions with ABPV RNA the primers were removed. The primers used for ABPV amplification were also changed at a later stage during the development of the multiplex PCR to the primer sequence as described in chapter two. Stoltz *et al.* (1995) and Evans, (2001) have both shown that KBV and ABPV can readily be distinguished by reverse transcriptase (RT) PCR in separate reactions with primers designed within the RNA dependant RNA polymerase (RdRp) gene of these viruses. Our strategy was to design the primers for the multiplex PCR within the capsid coding region of all of the viruses included in the PCR. The strain of KBV used in the multiplex PCR had not been sequenced to date, therefore it was decided to sequence a region of the virus to determine its homology with other KBV strains and also ABPV with which it interacted in the PCR. A 2 kilobase fragment of KBV was sequenced within the

capsid coding region. This chapter will determine the homology of KBV-in with other KBV strains. It will also consider whether the homology between this KBV strain and ABPV is high enough to cause the primer and template interactions seen during the previous study.

### **3.3 Materials and Methods**

#### **3.3.1 Virus propagation and isolation**

KBV (Indian strain) was kindly provided by Brenda Ball (Hamstead UK) and was used for the propagation and isolation of virus particles and RNA. KBV virus was injected into apparently healthy white eyed to pink eyed honeybee worker pupae collected from hives and left to incubate for 5 to 6 days in total at 30°C. The pupae were homogenised in batches of 10 in a mortar and pestle with 10 ml 0.01M phosphate buffer pH 7. The homogenate was mixed with carbon tetrachloride and diethyl ether and centrifuged for 10 min at 8000 rpm. The supernatant was then subjected to differential centrifugation. A sucrose gradient to isolate the virus particles was completed by layering onto a 10-40% (w/v) sucrose gradient and centrifuged in a SW 80 swing bucket rotor. The virus was isolated from the 30% sucrose band and centrifuged at 47 000 rpm. The virus particles were resuspended in 0.01M phosphate buffer pH 7 and stored at -80°C until used.

### 3.3.2 RNA extraction and cDNA synthesis

Total RNA was extracted by standard methods and precipitated by ethanol (Sambrook *et al.*, 1989). The extracted RNA was run on a 0.8% gel and quantified before continuing with cDNA synthesis using the Universal Riboclone cDNA synthesis kit (Promega). The poly T primer provided with the kit was used following manufacturers instructions to complete the synthesis reaction. RNA at a concentration of 0.5 mg/ml was used in the reaction with 0.5 mg/ml poly (T)<sub>15</sub> primer in order to synthesise cDNA. A total of three cDNA clones ranging from 400 bp to 1 kb were all cloned into pBluescript vectors (Stratagene) and transformed into competent JM109 *E. coli* cells. Primer walking was employed to obtain the three overlapping cDNA clones.

### 3.3.3 Reverse transcription PCR reactions

The PCR was carried out by adding the reagents to ABPV RNA with the primers designed to amplify from the KBV genome. The primer sequence was KBVF 5' ACT GTG GCA GCC ATC TTT GGA TG I 3', KBVR 5' TCA GTC GTT TTC CAG GTG AGG AC I. The second reaction contained KBV RNA with the primers to amplify the ABPV genome. The primer sequence for ABPV was ABPVF 5' GTA GCA TCT ACA ACC GAC AAA GG I 3', ABPVR 5' GAG GGT ATG TCT GTC CTC TAA AG I 3'. The I at the end of the primers sequences denotes an added inosine. PCR conditions used were described in chapter two. The PCR products were run on a 3% agarose gel. A 247 bp fragment was expected using the KBV primers and 200 bp fragment for the ABPV primers.

### 3.3.4 Sequence alignments

All of the nucleotide sequences obtained from Genbank were used in alignments. The following accession numbers were used for KBV from Canada (KBV-can AY452696), KBV from Pennsylvania (KBV-penn AY275710) and ABPV from the United Kingdom (ABPV-uk AF150629). The alignments were completed using MAFFT sequence analysis tool with the European bioinformatics institute at ([www.ebi.ac.uk](http://www.ebi.ac.uk)) from there the alignments were entered into a boxshade program at the Swiss institute of bioinformatics ([www.ch.embnet.org](http://www.ch.embnet.org)).

## 3.4 Results

### 3.4.1 Sequence alignments of KBV-in with other KBV strains

A 2 kb partial sequence of the KBV-in strain was determined by sequencing. The sequence was within the capsid coding region of the virus. Alignments of the sequenced KBV-in strain were completed against full genome KBV-penn and full capsid region KBV-ca. It has been reported that KBV has a wide diversity in homology between KBV isolates from different geographical regions (de Mirande *et al.*, 2004). This has impacted on the homology between the strains. The homology between KBV-ca and KBV-penn was higher than 95% at the nucleotide level. The KBV-in strain was seen to have an average of 72% homology over the 2 kb region when compared to KBV-ca and KBV-penn at the nucleotide level. In figure 3.1 the protein sequences are aligned to show the homology at this level.

```

KBV-penn 112 VLKAGGKAOKLANFKYLRCDVQVKIVLNANPFIAGRLYLAYSPLYDDKVAPEPERRIITYTSRA
KBV-ca 1 -----NFKYLRCDVQVKIVLNANPFIAGRLYLAYSPLYDDKVAPEPERRIITYTSRA
ABPV-uk 121 VLSAGGKGOKLANFKYLRCDVQVKIVLNANPFIAGRLYLAYSPLYDDKVAPEPARRIINTSRA
KBV-in 1 -----NFKYLRCDIQVKIVLNANPFIAGRMVAYSPLYDDKVAPEPARRIINTSRA

KBV-penn 172 GVTGYPGVELDFQLDNSVEMTIPYASFQEAIDLVSQNEDFVQLYLFTIAPVVLGPSAESAN
KBV-ca 49 GVTGYPGVELDFQLDNSVEMTIPYASFQEAIDLVSQTEDFVQLYLFTITPVLGPSAESAN
ABPV-uk 181 GVTGYPGTEIDFQLDNSVEMTIPYASFQEAIDLVTGTEDFVQLYLFTITPILSPITSTAS
KBV-in 49 GVTGYPGVELDFQLDNSVEMTIPYASFQEAIDLVKGTEDFVQLYLFTITPVLGPSAQTAQ

KBV-penn 232 SKVDLSVYMWLDNISLVIPTYRLN----PNLPTGQTLTRIVQNSDSDKLEALKIAKSKN
KBV-ca 109 SKVDLSVYMWLDNISLVIPTYRLN----PNLPTGQTLTRIVQNSDSDKLEALKIAKSKN
ABPV-uk 241 SKVDLSVYMWLDNISLVIPTYRNTSIVPNVGVTVVQTVQNMTRDSEITRKAVVALRKNN
KBV-in 109 SKVDLSVYMWLSNISLVIPTYRLN--SDIVKMATDPNINSVCWSSCRCKVYNRRSENPERK

KBV-penn 288 PSGYKYIMGVLEQYNPSVKQVSMQIATPNKSKS-----TKPTSSENPKIGPIS
KBV-ca 165 PSGYKYIMGVLEQYNPSVKQVSMQIATPNKSKS-----TKPTSSENPKIGPIS
ABPV-uk 301 KSTYDYIQALSSAVPEVKNVTMQINSKKNNSNKMATPVKEKTKNIPKPKTENPKIGPIS
KBV-in 168 SIWYQIYYECLDRLCTRSEKMPQVNRNNAKTKP-----VQKSTKPTSSENPKIGPIS

KBV-penn 335 EVASGVKTAANGIERIPVLGEIAKPVTAAVKWFADIVGGVAAIFGWSKPRNQNVMPYQN
KBV-ca 212 EVASGVKTAANGIERIPVLGEIAKPVTAAVKWFADIVGGVAAIFGWSKPRNQNVMPYQN
ABPV-uk 361 ELAIGVKNVANGIERIPVLCEMAKPVTSITKWWADKIGSVAAIFGWSKPRNLEQVNIYQN
KBV-in 221 EVASGVKTAANGMNVSQWVKLQSQ-----QPLSGELMLSELWQPSLDGPNPVIKIK

KBV-penn 395 VPGWGYSLYKGIDMSVPLADPNNELGDLRDVFPSSAVDEMAIGYVCGNPAIKHVLTSWIT
KBV-ca 272 VPGWGYSLYKGIDMSVPLADPNNELGDLRDVFPSSAVDEMAIGYVCGNPAIKHVLTSWNTT
ABPV-uk 421 VPGWGYSLYKGIDMSVPLADPNNELGDLRDVFPSSAVDEMAIGYVCGNPAIKHVLTSWNTT
KBV-in 272 CHIKMFLDGDILSIRELAFHLLTTLIMNIVTMMYFLOVLTKWLVMFAAILLSNMSPPGKR

KBV-penn 455 DVVQNPISNGDDWGGVIPVGMPCYSKTIKAVKCATSTSKTEVMDPAPCEYVANLFSYWRA
KBV-ca 332 DAVQNPISNGDDWGGVIPVGMPCYSKTIKAVKGDSTSTSKTEVMDPAPCEYVANLFSYWRA
ABPV-uk 481 DKVQAPIKSNNGDDWGGVIPVGMPCYSKTIKAVTENDTTRINTEIMDPAPCEYVCFNFSYWRA
KBV-in 332 LILQKPIANGDDWGGVIPVGMPCYSKHG-----SQWDAAPPWNMLPNKIFILACQTHWC

KBV-penn 515 TMCYRITVVKTAFTGRLEIFFEFGSIPTVRTADNLGPDQTLNGTIAPSDNNYKIYLLD
KBV-ca 392 TMCYRITVVKTAFTGRLEIFFEFGSIPTVRTADNLGPDQTLNGTIAPSDNNYKIYLLD
ABPV-uk 541 TMCYRIATVVKTAFTGRLEIFFEFGPKIPIITTTKDNISPDITQLDGIKAPSDNNYKIYLLD
KBV-in 386 LSEITVGVKNVSVSILADFEIFFVNPGDIPVKSCHCOLALNQDALFG-----RWLLPPI

KBV-penn 575 TNDTEVTIKVPPYVSNKMFMTVGIYGAHDEDNWDFDESFTGFCLCIRPITKLMAPDTVSOK
KBV-ca 452 TNDTEVTIKVPPYVSNKMFMTVGIYGANDENNWDFDESFTGFCLCIRPITKLMAPDTVSOK
ABPV-uk 601 TNDTEITIRVPPVSNKMFMTSTGIYGCNSENNWDFSESFTGFCLCIRPITKFMCPPTVSNIN
KBV-in 437 IIISTFWIRMLRQLEFPVYQIRCFSR-----LLVSMVLIVKITGTF

KBV-penn 635 VSIIVVWKAEDVVVVEPKPLTSGPTQVYNPPAVARDLVKQIDVSMQINLSNKTDENTISF
KBV-ca 512 VSIIVVWKAEDVVVVEPKPLTSGPTQVYNPPAVARDLVKQIDVSMQINLSNKTDENTISF
ABPV-uk 661 VSIIVVWKAEDVVVVEPKPLTSGPTQVFQPPVTSADSNITIDASMQINLANKADENVVIF
KBV-in 479 KNPVVDSEVLDQLNWLPKCLTI-----

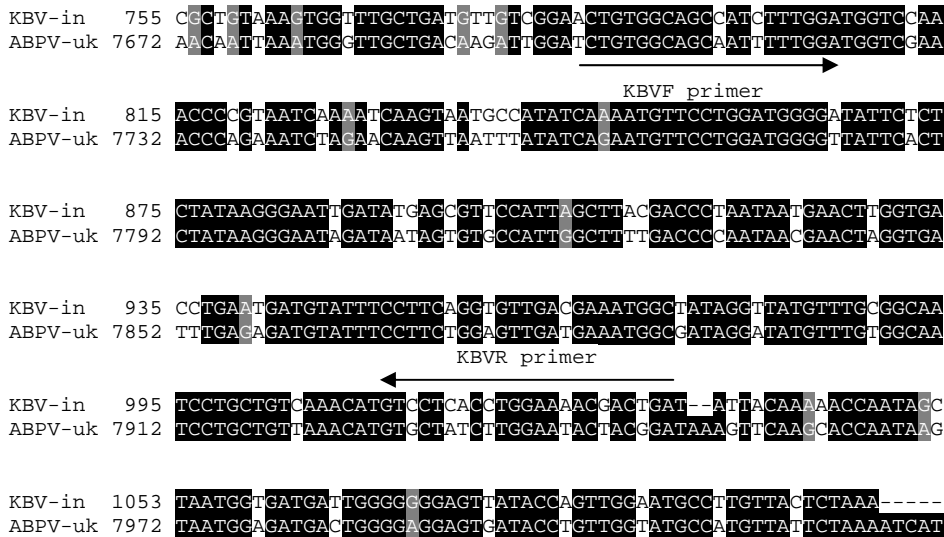
```

**Figure 3.1**

Protein sequence alignments of different KBV strains and ABPV covering the capsid coding region of these viruses. The protein sequence of the capsid regions of KBV-penn, KBV-ca, KBV-in and ABPV were aligned to show homology between these regions of different strains of KBV and related virus ABPV. The line under the sequence indicates the conserved picorna-like virus capsid protein domains 1 and 2.

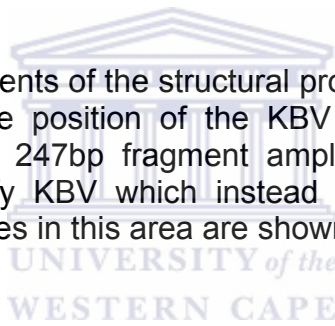
### 3.4.2 Sequence alignments of KBV with ABPV and primers designed

With the sequencing of the full genome of KBV-penn it was observed that KBV exhibits elements that are different to ABPV in certain areas (de Mirande *et al.*, 2004). Therefore the suggestion that KBV and ABPV is the same virus cannot be supported. However there are regions within KBV and ABPV genomes which are highly homologous. Primers were designed previously to amplify KBV and ABPV in a multiplex RT PCR (chapter two). KBV was subsequently removed from the primer pool used in the multiplex PCR. The removal of KBV was prompted by KBV and ABPV primers amplifying products from incorrect templates. To investigate the reasons for this the primers designed for KBV and ABPV were aligned. Figure 3.2 shows that the KBV primer and ABPV primer are highly homologous to the other sequence. The primers are approximately 81% homologous. The high homology was not initially observed when primers were designed. Primers used in the multiplex RT PCR as described in chapter two fell outside of the 2 kb region of KBV-in which was sequenced and therefore was not included in the alignment.



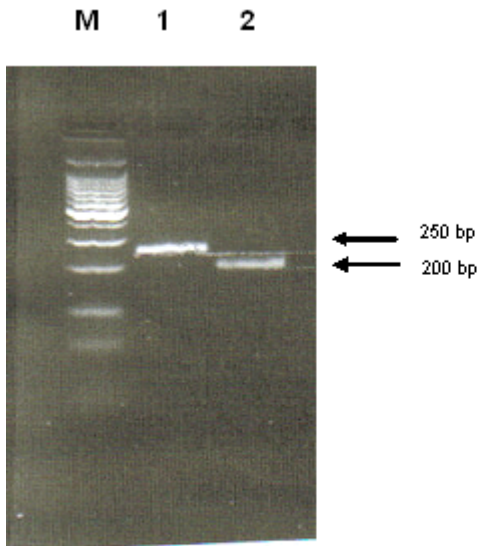
**Figure 3.2**

Nucleotide sequence alignments of the structural protein of ABPV-uk and various strains of KBV showing the position of the KBV primers. The area shown between the arrows is the 247bp fragment amplified with KBVF and KBVR primers designed to amplify KBV which instead amplified ABPV. The high homology between the viruses in this area are shown.



### 3.4.3 RT PCR reactions

RT PCR reactions were completed to demonstrate the interaction between the primers and templates added to the PCR. As described in material and methods the KBV primers were added to the ABPV template and the ABPV primers to the KBV template. In figure 3.3 it can be seen that the primers amplified the template present even though it was not the corresponding template RNA. The products were amplified at the correct fragment sizes for each reaction. The products were not however sequenced.



**Figure 3.3**

Detection of viruses with primers in laboratory infected honeybees. The primers interacted causing amplification of the incorrect genome as indicated by amplicon sizes shown. Lane 1, ABPV RNA amplified with KBV primers. Lane 2, KBV RNA amplified with ABPV primers. Lane M, 100 bp marker (Promega).

### 3.5 Discussion

Many studies have been conducted on KBV and ABPV because of their close relationship (de Mirande *et al.*, 2004, Allen & Ball 1995). Previously only short sequences of the KBV genome were available for alignments and phylogenetic analysis. Until recently when a strain from Pennsylvania USA was completely sequenced as was the coat protein gene from a KBV strain from Canada. The areas of homology between ABPV and KBV across the entire genome were shown and also the differences in very critical areas of the genomes (de Mirande *et al.*, 2004).

In this study a 2 kb cDNA fragment was cloned and sequenced. The sequence was from the 3' end, or capsid coding region of the virus. Alignments with other KBV sequences available showed homology in conserved areas of the capsid region. Geographically diverse strains were not highly homologous over the area that was concentrated upon. However strains from the same areas are more highly homologous than those found in other regions (de Mirande *et al.*, 2004). At both the nucleotide and protein level the virus strains displayed variability in areas which are not conserved. Further study into the variance in genomes of KBV strains found in different areas would be beneficial.

The KBV-in strain was also aligned with ABPV over the genome area where the primers were designed to amplify KBV in a multiplex RT PCR. This was done due to the primers amplifying a product even in the absence of KBV RNA. The area used to design primers for KBV in a multiplex RT PCR was highly homologous to ABPV which lead to the primers amplifying ABPV instead of the

target virus being KBV. The KBV RNA and primers were later removed from the assay. The primer selection used for typical assays like RT PCR are thus critically important for specificity of the PCR assay, observed in this study and in a similar study by Chen *et al.* (2004).

In this chapter and chapter two, laboratory infected pupae were used to optimise a multiplex RT-PCR before being tested on field samples which could have multiple inapparent viruses. Chen *et al.*, (2004) only used naturally infected field samples with the result that ABPV primers were removed from the primer pool in this study. The reason ABPV was removed in this study was that no ABPV was amplified in any of the samples. Whether this was due to ABPV not being present, which seems unlikely, in these samples or that the primers did not perform sufficiently within the multiplex used directly on field samples was not answered by this study.

This chapter has shown that the KBV-in strain is not highly homologous at the protein level to KBV-penn and KBV-ca which could be due to geographical variance. Also KBV-in is highly homologous to APBV in a region which was used to design primers for a multiplex RT PCR. Further sequencing of this strain could show the extent of the variance with other KBV strains from around the world. Also further sequencing of strains from other parts of the world would assist in the development of further detection systems.

## CHAPTER FOUR

### Expression of Virus like particles of honeybee virus Black Queen Cell Virus, by a heterologous baculovirus expression system

#### 4.1 Abstract

Black queen cell virus (BQCV), a member of the virus family *Dicistroviridae*, was identified as having four coat proteins in the second open reading frame of the virus genome. A PCR product of the structural genes was cloned under the control of a baculovirus P10 promoter in a transfer vector pAcAB4. Expression of the coat proteins by a heterologous baculovirus expression system in *Spodoptera frugiperda* (Sf 21) cell culture produced virus like particles (VLPs) of BQCV. The VLPs were isolated from a 10% sucrose gradient after ultracentrifugation of the cell suspension. Electron microscopy showed that the proteins had self assemble into 30nm particles which were structurally similar to the wildtype BQCV virus. The expression of BQCV VLPs proteins was verified by polyacrylamide gel electrophoresis and was compared to the wildtype BQCV coat proteins. Finally confirmation by western blot analysis was completed with antibodies raised to BQCV.

## 4.2 Introduction

The recently named virus family *Dicistroviridae* (genus *Cripavirus*) is composed of positive sense single stranded RNA viruses that infect a wide range of different insect hosts. These viruses were previously classed as cricket paralysis like-virus (Mayo, 2002). Honeybee virus, black queen cell virus (BQCV) previously classed as cricket paralysis-like virus has been included into this new family based on its biophysical features and genome properties (Leat *et al.*, 2000; Mayo, 2002). These viruses consist of two open reading frames which contain the non structural genes at the 5' end and structural genes at the 3' end of the virus.

BQCV was first isolated from queen larvae and pupae of honeybees by Bailey & Woods (1974). It was found to infect adults and pupae of honeybees producing symptoms where bee pupae darkened (Scott-Dupree & McCarthy, 1995). This virus is often present in bees that are infested with the microsporidian mite *Nosema apis*. The mite has been implicated in the mortality of bees infected with the virus and parasite simultaneously (Allen & Ball, 1996; Bailey *et al.*, 1983). The BQCV genome consists of single stranded RNA consisting of 8550 nucleotides and two open reading frames (ORFs). As with other viruses in this family, BQCV has been suggested, to have an internal ribosome entry site (IRES) starting at a CCU codon (Leat *et al.*, 2000).

Honeybee viruses were discovered some 30 years ago and to date only a few of the 18 known viruses have been sequenced. Restricted molecular based experimentation has been done on bee viruses because the virus cannot be

propagated in a suitable cell culture system. The traditional or manual propagation methods of honeybee viruses are all time consuming and labour intensive. Adult bees have to be collected and virus laden food is fed to adults. Alternatively pupae are collected and manually injected with virus preparations (Bailey & Woods, 1974, 1977). Due to inapparent viruses, which persist in the bee, there are uncertainties about whether the virus which was injected will be the virus that is propagated. It has been found in many cases that mixed infections occur when viruses are propagated in bees by traditional methods and have been reported previously (Evans, 2001). There has been no alternative host or cell line found for propagating honeybee viruses to date. Until now honeybee viruses have been detected by immunoblots, ELISA and more recently PCR based detection of viruses (Anderson, 1984; Anderson & Gibbs, 1988; Stoltz *et al.*, 1995; Benjeddou *et al.*, 2001; Chen *et al.*, 2004; Topley *et al.*, 2005). Previous tests which included antibodies were important for virus detection at the time, however due to the presence of mixed infections which we now know occurs, the antibodies used to detect and identify particular viruses are questionable.

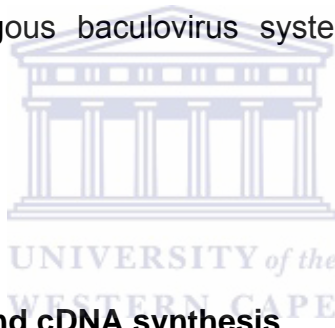
The sequencing of BQCV indicated that the viral capsid proteins consists of four proteins in the second ORF with sizes of 34, 32, 29 and 6kDa for CP1 to CP4 respectively, which makes up the isometric 30nm particle virus (Leat *et al.*, 2000). Recently an infectious virus of BQCV was developed by Benjeddou *et al.* (2002), which was found to be as infectious as the wild type virus, however

having a cell culture system would have increased the potential and ease for further study using this infectious virus.

Many RNA viruses lacking tissue culture systems have used recombinant baculovirus technology because of its convenience and proven output in expression of various protein products. The advancement in gene manipulation and also at expressing foreign genes in heterologous systems has proven indispensable (Belyaev & Roy, 1993). Compared to the prokaryotic systems used in the past, baculovirus expression systems allow for the synthesis of various proteins, posttranslational proteolytic processing and cleavage of signal peptides which would be performed naturally in the host (Beljelarskaya, 2002). *Autographa californica* nuclear polyhedrosis virus (*AcNPV*) polyhedron (ph), P10 and immediate early 1 (IE1) promoters have been widely used in the development of many different vector systems (Pullen & Friesen, 1994; Huynh & Zieler, 1999; Belyaev & Roy, 1993). These three constitutive promoters are advantageous in that they produce large amounts of foreign gene products during infections (Jarvis *et al.*, 1996). Baculovirus systems have been used to express virus like particles (VLPs) of many different viruses including blue tongue virus (Belyaev and Roy 1993), cowpea mosaic virus (Shanks & Lomonosof, 2000), African horsesickness virus (Maree *et al.*, 1998), SARS coronalike virus particles (Mortola & Roy, 2004) and Norwalk virus (Jiang *et al.*, 1992). The promoters have also shown to have a wide range of compatibility within other host cells other than the traditional *Spodoptera frugiperda* (*Sf*) 9 or *Sf* 21 cells. This was shown when hepatitis C virus expressed in hepatocyte derived cell lines

(McCormick *et al.*, 2002) and various proteins in mosquito cell lines using baculovirus promoters (Huynh & Zieler, 1999). The expression of proteins by these systems has allowed for the further understanding of virus capsid proteins, how they assemble and also the expression of other proteins.

The capsid structure of BQCV and other honeybee viruses have not yet been characterised in detail and not much is known about virus interaction or capsid construction. To date no honeybee virus has been used in a heterologous system to express VLPs to our knowledge. Therefore the aims of this study are to observe whether BQCV capsid proteins are able to assemble into virus like particles using a heterologous baculovirus system expressed in insect cell culture.



### **4.3 Materials and Methods**

#### **4.3.1 BQCV propagation and cDNA synthesis**

BQCV was propagated as described by Leat *et al.*, (2000) (chapter 2) and virus isolation was stopped after the sucrose gradient step. RNA was extracted from the isolated virus using the SV Total RNA isolation kit (Promega) as described by the manufacturers. cDNA was synthesised by using the Universal RiboClone cDNA synthesis kit (Promega). The reverse transcription reaction was carried out in a total volume of 25µl which included viral RNA (2 µg) with 5 µg/ml of gene specific primer FCDNA 5' TTTTTTTTTTTTTTGCAAC 3' (Benjeddou *et al.*, 2002). The cDNA reaction was completed as per manufacturer's recommendation except that the incubation of first strand cDNA was 4 hours at 42°C and the

synthesis was stopped after the first strand. Following this 1  $\mu$ l of RNase H (2 units/ $\mu$ l) was added and the reaction incubated for 20 min at 37°C (Benjeddou *et al.*, 2002).

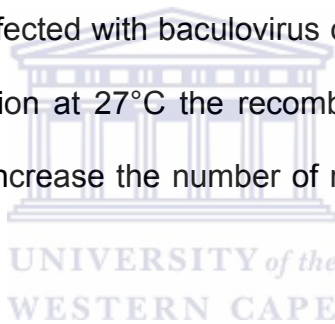
#### 4.3.2 PCR of capsid gene

Primers were designed to the entire capsid coding region from the start to the stop codon for the capsid gene. The primers used to amplify the capsid gene were designed from the sequence in the NCBI database accession number AF183905, CAPF 5' GGG TAT AGA TCT **ATG** CCT GCT GAA CAA ATT AAT GAA 3' (position 5834-5853) and CAPR 5' GGG TAT AGA TCT TCA CAA CAA ATC GCT ATC C 3' (position 8376-8395). The underlined sequence represents the *Bgl* II restriction sites and the bold represents the start codon incorporated into the sequence by PCR for transcription initiation. The PCR was carried out in a total volume of 50 $\mu$ l with 2 $\mu$ l of cDNA as template using the Expand high fidelity PCR system (Roche) as recommended by the manufacturer. The resulting PCR fragment was gel purified using the High pure PCR purification kit (Roche) and ligated into the pGem T vector (Promega) resulting in pGemCAP. The amplified capsid gene was then sequenced to ensure the capsid protein and restriction sites were correct before proceeding with further subcloning. The baculovirus transfer vector pAcAB4 (Pharmingen) was digested with *Bgl* II as well as pGemCAP. The resulting *Bgl* II digested pAcAB4 and PCR excised gel fragment were purified by using the High pure PCR purification kit (Roche). The capsid

gene was then subcloned into the *Bgl II* site of the pAcAB4 plasmid under the control of the p10 promoter resulting in the transfer vector pAcCAP.

#### **4.3.3 Recombinant baculovirus production and VLP expression**

*Sf 21* cells were grown up in TC-100 insect media (Sigma) and maintained at 27°C. The calcium phosphate mediated co-transfection method was used to co-transfect pAcCap with the BD baculo gold linear DNA (Pharmingen). Once the cells reach 50-70% confluence 0.5 µg linear DNA and 2.5 µg pAcCAP was added to the cells as per the recommendations of the manufacturer. A negative control consisted of *Sf 21* infected with baculovirus only and left to incubate for 2 days. After 2 days incubation at 27°C the recombined virus was infected into fresh *Sf 21* cells to further increase the number of recombinant virus and grown for a further 48 hours.



#### **4.3.4 SDS polyacrylamide gel electrophoresis**

The supernatant from the cell suspension containing the VLPs were incubated in lysis buffer 0.5 M Tris-HCL pH 6.8, 1% β-mercaptoethanol, 10% sodium dodecyl sulfate (SDS), 20% glycerol and 1% bromophenol blue and incubated at 95°C for 5 min. The proteins were resolved on a 12% SDS polyacrylamide gel with the stacking gel run at a voltage of 80V/m and the resolving gel at 100V/m. Prestained protein molecular marker (Fermentas) was used to determine the sizes of the proteins. To visualise the proteins the gel was stained with Page Blue protein staining solution (Fermentas).

#### **4.3.5 Western blot analysis**

A separate SDS PAGE was run for the western blot with the Benchmark pre-stained protein ladder (Invitrogen). The proteins were transferred onto PVDF western blot membrane (Roche) which was pre-soaked in methanol for 4 seconds and then rinsed in sterile distilled water for 1-2 min. The proteins were transferred at 300mA for 2 hours in 1X transfer buffer (10X Tris borate EDTA and 20% methanol [v/v]). The BQCV capsid proteins were identified by probing with rabbit polyclonal antibodies against purified BQCV at a ratio of 1:2000 in 3% skim milk as a primary detection at 4°C overnight. The blot was then washed three times with Tween PBS (Tween 20, 1X PBS). The secondary detection was completed at a ratio of 1:2000 with goat anti-rabbit antibodies in 3% skim milk for 1 hour at room temperature. The blot was washed in Tween PBS three times and positive signals were visualised with 3, 3', 5, 5'-tetramethylbenzidine (TMB) membrane peroxidase substrate (KPL).

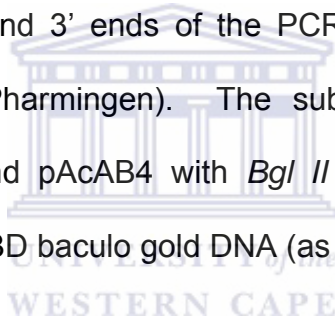
#### **4.3.6 Electron microscopy**

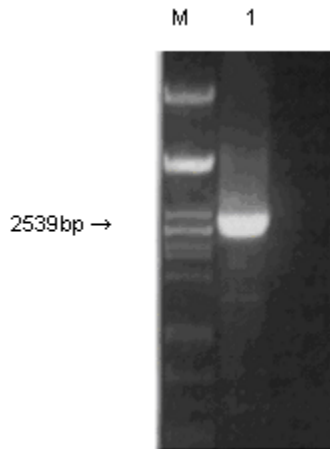
The expressed VLPs in the cell suspension was centrifuged on a discontinuous sucrose gradient of 10-40% (w/v) in 0.01M phosphate buffer at 27 000 rpm for 2 hours in a SW80 swing out bucket rotor. Fractions were collected at the 10, 20 and 30% interface. The VLPs were then absorbed onto carbon coated copper mesh and washed with distilled water. The particles were stained with 2% uranyl acetate and examined by transmission electron microscope.

## 4.4 Results

### 4.4.1 Construction of transfer vector

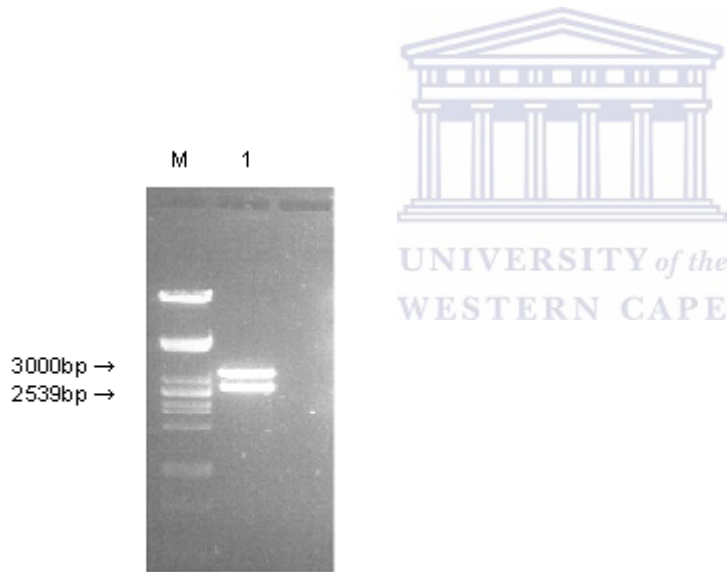
The full length single stranded cDNA synthesised from BQCV RNA was used unpurified in a PCR with the CAPF and CAPR primers. The amplified product was run on a 0.8 % agarose gel to confirm the amplicon size (figure 4.1). It was then cloned into pGem T and sequenced to ensure that the entire capsid gene had been amplified, that the restriction sites were incorporated and that the initiation codon was present with no nucleotide changes. To insert the BQCV capsid gene into the genome of AcNPV the PCR product which included *Bgl* II restriction sites on the 5' and 3' ends of the PCR product was cloned into a transfer vector pAcAB4 (Pharming). The subcloning was completed by digesting the pGemCap and pAcAB4 with *Bgl* II (figure 4.2). pAcCAP was recombined with linearised BD baculo gold DNA (as described in methods).





**Figure 4.1**

Amplification of the BQCV capsid gene with full length single stranded (ss) cDNA. Primers CAPF and CAPR introduced an ATG for initiation of replication in cell culture. M *Pst* lambda DNA marker; lane 1 BQCV capsid PCR product.

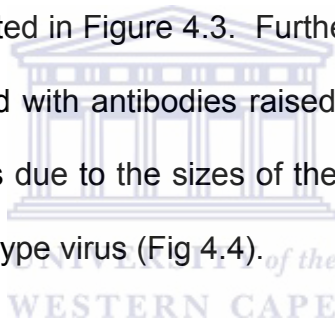


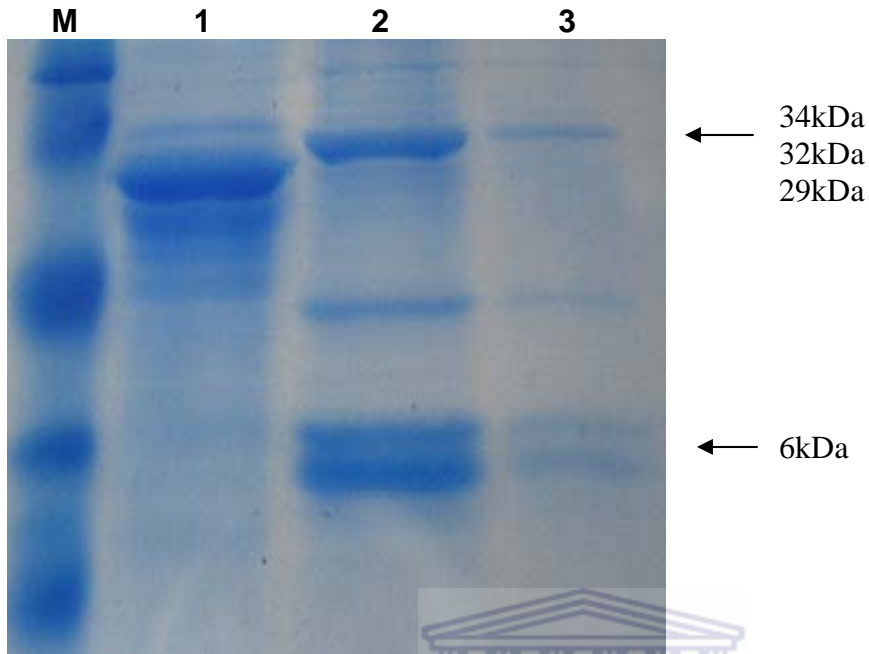
**Figure 4.2**

Plasmid DNA isolation and restriction digest of pGemT containing the BQCV capsid gene. The resulting pGemCAP plasmid was digested with *Bgl II*. Lane M, *Pst* lambda DNA marker; lane 1 pGem vector (3000bp) and BQCV capsid gene (2539bp);

#### **4.4.2 Expression of BQCV virus-like particles in baculovirus**

The baculovirus transfer vector pAcCAP and linear baculovirus DNA was co-infected in the insect cell line *Sf 21*. The insect cells infected with the recombinated baculovirus containing the BQCV capsid protein was incubated at 27°C for two days. The BQCV VLPs were expressed by the infected cells and released into the surrounding insect media. The medium and cells were harvested after the two days. The supernatant was separated from the cells by centrifugation and the expressed VLPs in the supernatant were collected. The VLPs were subjected to SDS PAGE and the four capsid proteins of the BQCV virus were present as indicated in Figure 4.3. Further more western blot analysis of the coat proteins detected with antibodies raised against BQCV showed they were indeed BQCV particles due to the sizes of the proteins detected and when compared to the BQCV wildtype virus (Fig 4.4).

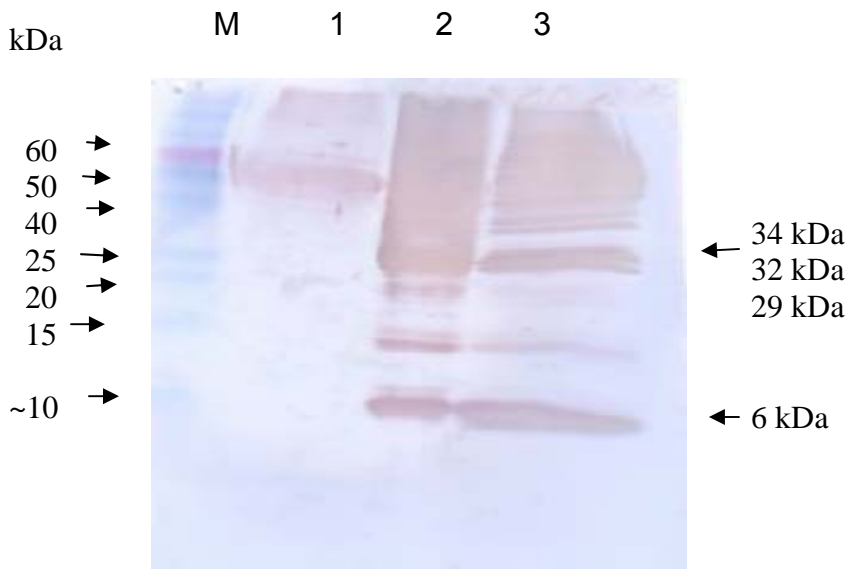




**Figure 4.3**



SDS-PAGE analysis of BQCV VLPs expressed by heterologous baculovirus expression. Virus like particles (VLPs) were obtained by expressing BQCV capsid genes under the p10 promoter of pAcAB4 vector in a *Sf* 21 cell line. Lane M, Pre-stained protein molecular weight marker (Fermentas); lane 1, negative control of cells infected with baculovirus only; lane 2, BQCV wild type virus; lane 3, shows the four expressed capsid proteins produced by heterologous baculovirus expression. The, 34, 32 and 29kDa bands appear as a triplet in the top band.



**Figure 4.4**

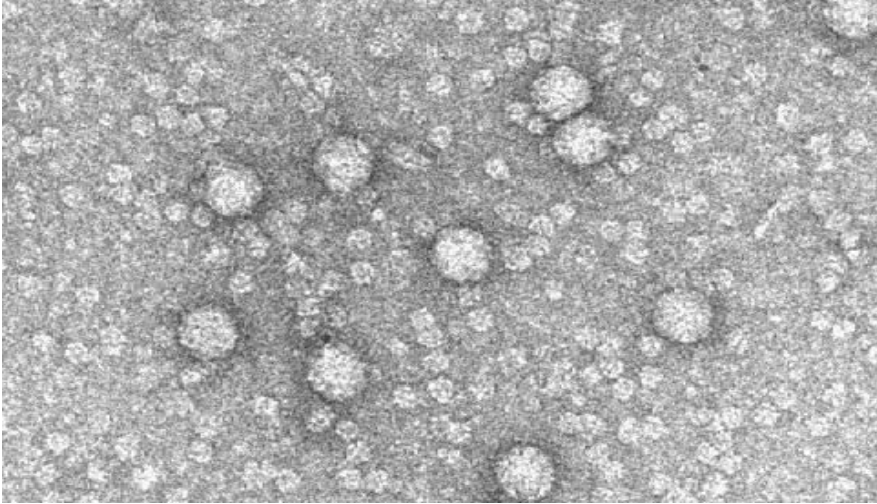
Western blot analysis of the wild type capsid proteins of BQCV and heterologous expressed virus like particles (VLPs) of BQCV virus. Wild type virus and VLPs expressed by recombinant baculovirus were detected with antibodies raised to BQCV. The wild type virus and VLPs were separated on a sucrose cushion and concentrated by ultra centrifugation. Lane M, Invitrogen bench mark prestained protein marker; lane 1, insect cells infected with baculovirus only (negative control); lane 2, BQCV wild type virus capsid proteins (positive control); lane 3, shows the four capsid proteins produced by heterologous baculovirus expression which make up the VLP.

#### 4.4.3 Electron microscopy of VLPs

The insect media supernatants were examined using transmission electron microscopy to determine if VLPs were produced by heterologous expression. wtBQCV capsids were compared to the VLPs produced by the recombinant baculovirus. Figure 4.5A shows the wildtype (wt) BQCV capsids containing nucleic acid. This was compared to the capsids produced by the recombinant baculovirus expressing VLPs of BQCV in cell culture in figure 4.5B. The buoyant density of the empty capsids in the sucrose gradient also differed to the capsids containing RNA in that the empty capsids were isolated at the 10% gradient compared to 30% for the wtBQCV capsids.



A



B



**Figure 4.5**

Transmission electron micrographs of BQCV virus particles. Bee pupae were injected with BQCV virus as described in methods for the wild type controls and BQCV virus like particles (VLPs) were produced by heterologous expression of the capsid gene in baculovirus cell culture. (A) wild type BQCV recovered from honeybee pupae injected with virus for propagation showing full capsids. (B) empty capsid shells composed of the four coat proteins of BQCV expressed in insect cells infected with the pAcCAP vector.

## 4.5 Discussion

A PCR product of the capsid coding region of honeybee virus BQCV was cloned into a baculovirus transfer vector pAcAB4 under the control of the P10 promoter. Previous sequence analysis and protein sequencing showed that the structural genes cleaved to produce four coat proteins that form the capsid of the BQCV virus (Leat *et al.*, 2000). The coat proteins were expressed by the transfer vector in an insect cell line *Sf 21* to form the VLPs. After centrifugation on a sucrose cushion the VLPs were found to be in the 10% sucrose layer. This is in contrast to the previously propagated virus capsids which were usually isolated from the 30% sucrose layer. The VLPs observed by electron microscopy appeared to be structurally similar to the wtBQCV capsid proteins when compared (fig 4.5B). Expression of VLPs for a honeybee virus in a heterologous system has not to our knowledge been carried out previously and no examples exist to compare our findings. Similar results were seen however when the virus *Thosea asigna* was expressed in a baculovirus expression system where the capsids were also structurally similar to the wild type virus (Pringle *et al.*, 2001). Confirmation of the expressed proteins which forms the VLPs was done by SDS-PAGE and western blots which showed the relevant proteins representing the four capsid proteins of the BQCV virus.

Heterologous systems for expression of foreign genes have been widely used with baculoviruses having been proven to be an excellent system in which to express foreign genes (Huynh & Zieler 1999; McCormick *et al.*, 2002; Medin *et al.*, 1990; Mortola & Roy, 2004). Baculovirus systems have been used to

express widely different species of virus proteins many of which do not have cell cultures available and the proteins have been expressed successfully. Using the baculovirus expression systems more studies could be conducted on viruses than without a cell culture system. Such studies would include defining exactly how capsid expression takes place, which proteins are involved in capsid formation and how to prohibit the formation of capsids in viruses (Pringle *et al.*, 2001; Maree *et al.*, 1998; Shanks & Lommossoff 2000). Having a pure culture of a particular honeybee virus can improve immune diagnostics used to identify honeybee viruses and raise specific antibodies to pure expressed and infectious viruses. With a cell culture system many extra preliminary steps in experiments carried out on honeybee viruses could be improved (Benjeddou *et al.*, 2002). The production of potential recombinant vaccines lacking infectious RNA can also be an alternative (Roosien *et al.*, 1990).

With naturally infected, inapparent or apparently healthy bees RT PCR methods have been used successfully to identify and detect viruses in bees that appeared to be infected or apparently healthy at even very low infection doses (Hung & Shimanuki, 1999; Stoltz *et al.*, 1995; Benjeddou *et al.*, 2001; Chen *et al.*, 2004; Topley *et al.*, 2005). Traditional methods used in the past and still being used to identify and also propagate viruses have their disadvantages. When propagation of the virus becomes necessary inapparent viruses has been shown to be present (Evans *et al.*, 2001; Anderson & Gibbs 1988). This could hinder experimental work in some cases to be completed with the propagated virus as it could be an impure virus isolate. Many identification methods for honeybee

viruses were based on antibody derived methods and having a mixed infected isolate could be of significance to the experiment.

In nature honeybee populations are being devastated due to colony collapse disorder by acute viral infections in many states within the US (Cox-Foster *et al.*, 2007). Therefore a propagation method for new or emerging viruses will have to be investigated to prevent further colony collapse in future. Production of pure and infectious virus by cell culture can therefore be used to raise antibodies to reliably detect specific viruses.

This heterologous baculovirus expression system produced VLPs of honeybee virus BQCV in *Sf* 21 insect cells. This system could be used in future to study the capsid assembly of BQCV or other honeybee virus more closely and furthermore BQCV could also be used as a model for honeybee viruses. The baculovirus system could also be used to produce pure infectious honeybee viruses as many commercial vectors have multiple cloning sites with promoters for three or four genes. This makes the possibility of expressing genes in combinations, entire viruses and even altered viruses very good.

## CHAPTER FIVE

### Construction of a chimeric honeybee virus

#### 5.1 Abstract

Black queen cell virus (BQCV) and acute bee paralysis virus (ABPV) were used in an attempt to fuse opposite open reading frames (ORFs) of these two viruses to produce a chimeric virus. The nonstructural genes in the 5' ORF of BQCV were to be exchanged with the structural genes in the 3' ORF of ABPV and vice versa. Full length single stranded cDNA was used in PCR reactions to amplify the specific ORFs. A fusion PCR strategy was then employed to join the halves of the viruses to form a chimeric honeybee virus. The chimeric virus would have been useful in observing any changes in virus infectivity and assembly. The non structural genes in the 5' open reading frame (ORF) and the structural genes in the 3' ORF were successfully amplified from both viruses. However the fusion PCR strategy used to join the genome halves was unsuccessful. Alternative strategies are suggested that could see better results in future.

## 5.2 Introduction

Black queen cell virus (BQCV) and acute bee paralysis virus (ABPV) are viruses that infect honeybees. These viruses can both cause decreases in colony size and on occasion death of these colonies. Many viruses have been isolated which infect honeybees but only a few cause high mortality rates. BQCV was first isolated from queen larvae and pupae of honeybees found dead in their cells (Bailey & Woods, 1974). The virus causes the cells containing the infected larvae to darken. It was observed that BQCV is often present in bees infested with the microsporidian parasite *Nosema apis* in UK hives. The relationship between virus and parasite is implicated in the mortality of bees infected with this parasite (Allen & Ball, 1996, Bailey *et al.*, 1983).

ABPV was discovered during infection experiments in the laboratory (Bailey *et al.*, 1963). The virus can be present as an inapparent infection when associated with colonies infected with the parasitic mite *Varroa destructor* (Allen & Ball, 1996; Ball, 1989; Ball & Allen, 1988). ABPV spreads by way of salivary gland secretions of adult bees. The virus makes its way into the broods' food stores and so the virus is spread throughout the colony (Ball, 1985). Many honeybee viruses persist as inapparent viruses and increasing knowledge in the relationship between the honeybee virus and the parasitic mite *V. destructor* has lead to many suggestions that these mites may be involved in honeybee mortality (Bailey *et al.*, 1983; Ball & Allen 1988; Allen & Ball, 1996; Brødsgaard *et al.*,

2000). The term bee parasitic mite syndrome has been used when a diseased complex exists in a colony where mites and viruses are present and there is a high mortality rate (Shimanuki *et al.*, 1994).

BQCV and ABPV are both 30nm particle viruses and have single stranded positive RNA genomes. The South African isolate of BQCV has an 8550 nucleotide genome and ABPV was found to have 9470 nucleotides excluding the poly (A) tails (Leat *et al.*, 2000, Govan *et al.*, 2000). The molecular masses of the four capsid proteins for each virus are; BQCV 34, 32, 29 and 6kDa (Leat *et al.*, 2000) and ABPV are 35, 33, 24 and 9.4kDa for VP1-VP4 (Govan *et al.*, 2000). Both BQCV and ABPV contain two open reading frames (ORFs) containing a 5' ORF encoding a putative replicase protein and a 3' ORF encoding a capsid polyprotein. Due to this orientation BQCV and ABPV had been added to the cricket paralysis-like or picorna-like virus group (Leat *et al.*, 2000, Govan *et al.*, 2000). This novel group of insect infecting viruses included 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). Recently all of these viruses have been re-classed as *Dicistroviridae* and genus Cripavirus (Mayo, 2002). This group of viruses has the same orientation of the replicase polyprotein and the capsid polyprotein as the two honeybee viruses. CrPV, DCV, PSIV, RhPV and HiPV are all monopartite bicistronic viruses. In the case of PSIV the initiation of translation in the proximal ORF has been demonstrated to be dependent on an internal ribosome entry site (IRES) starting at a non AUG site (Sasaki & Nakasima,

1999). Like PSIV it has also been suggested that BQCV translation initiation is facilitated by an IRES element at a CCU codon (Leat *et al.*, 2000).

Recently a stable infectious virus copy of BQCV was developed. The infectious virus was fully functional and able to infect honeybee pupae when injected and the virus could not be distinguished from the wild type virus by electron microscopy. The infectious virus was mutated to clearly distinguish it between the viral particles recovered from experiments originating from the infectious transcripts and not from an inapparent infection or wild type virus (Benjeddou *et al.*, 2002). The mutation in the infectious virus was introduced by fusion PCR that was a combination of methods used by Gritsun & Gould (1995) and Rebel *et al.* (2000). The principle of fusion PCR has also been employed in producing a chimeric virus, though not a method widely used, it has been shown however to be a simple technique to join two halves of two specific viruses to produce a chimeric virus (Dekker *et al.*, 2000).

Chimeric viruses are viruses that have been altered at the gene level by substituting different or corresponding regions between two or more viruses. The chimeric viruses have been shown to be very useful in the study of specific functions of viral components by interchanging or swapping regions (Kuhn *et al.*, 1996; Powers *et al.*, 2000). Chimeras have also been extensively used in vaccine studies to create possible vaccines from altered viruses (Pletnev & Men, 1998; Pletnev *et al.*, 1992; Igarashi *et al.*, 1997). In the vaccine studies the chimeric viruses were observed to be either more virulent or avirulent compared to the

parental virus strain and in many cases the results have been important in virus research.

In this study it was planned to construct a chimeric virus where portions of the genome were derived from one honeybee virus (BQCV) and the remainder of the genome derived from the second honeybee virus (ABPV). If the components from the two viruses could interact with one another a functional infectious virus would be obtained and the infectious ability of the virus could be studied. If this did not occur the chimeric construct was attenuated or nonviable. A chimeric honeybee virus would also better define the different contributions of viral components to virus growth and virulence.

### **5.3 Materials and Methods**

#### **5.3.1 Propagation**

ABPV and BQCV were propagated in apparently healthy white and pink eyed honeybee pupae. The pupae were injected with a viral suspension of either BQCV or ABPV as described in chapter two and incubated for 6 to 7 days at 30 to 35°C. The viruses were extracted and purified as described by Leat *et al.* (2000).

#### **5.3.2 RNA purification**

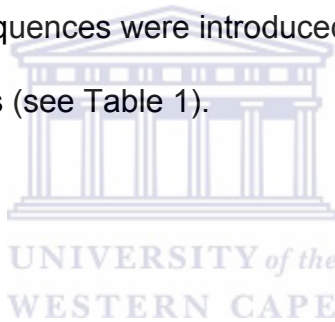
RNA was extracted from 100 µl virus suspension by using the SV total RNA isolation kit (Promega) following the manufacturers instructions. The RNA was eluted into 40µl of nuclease free water and the concentration was quantified by a



UV spectrophotometer and used immediately in the synthesis of first strand cDNA for both BQCV and ABPV.

### 5.3.3 Primers

Primers were designed on the basis of the nucleotide sequence of BQCV SA accession number AF 183905 and ABPV accession number AF 150629 (Table 1). Primers were designed for production of full length single stranded (ss) cDNA of both viruses, the PCR amplification of the specific 5' and 3' halves of the respective genomes and full chimeric viruses. The SP6 promoter and restriction enzymes *Not I* and *Kpn I* sequences were introduced into certain primers to allow for subsequent cloning steps (see Table 1).



**Table 5.1.** Sequence of primers used for the amplification of full length cDNA and genome halves of ABPV and BQCV

| Primer name | RE site     | Nucleotide sequence                                                                 | Genome position                      |
|-------------|-------------|-------------------------------------------------------------------------------------|--------------------------------------|
| 1           |             | 5' TTTTTTAAATTTACTAATTC 3'                                                          | 9462-9477                            |
| 2*          |             | 5' TTTTTTTTTTTTTGCAAC 3'                                                            | 8546-8563                            |
| 3           |             | 5' GGGTAACCATGTTGTGTTGCGATTCCCAA<br><u>CTACTCATAACCTGAAAGGCCAAGAGCAATC</u> 3'       | 6322-6355<br><u>5595-5628</u>        |
| 4           |             | 5' <u>GATTGCTCTTGGCCTTTCAGGTTATGAGTAGTT</u><br>TGGGAATCGCAACACAACATGGTTACCC 3'      | <u>5595-5628</u><br>6322-6355        |
| 5*          | <i>KpnI</i> | 5' GGGTAT <b>GGTACC</b> (T) <sub>25</sub> GCAACAAGAAGAAACGT<br>AAACC 3'             | 8529-8575                            |
| 6           | <i>NotI</i> | 5' GGGTATGCGGCCGC (T) <sub>40</sub> AATTTACTAATTCG<br>AAATTTTGACGC 3'               | 9445-9531                            |
| 7           | <i>NotI</i> | 5' GGGTAT <b>GCGGCCGC</b> ATTTAGGTGACACTATAGA<br>ATACCCGTCAAATAACAACCTTATAACAC 3'   | 1-26                                 |
| 8           |             | 5' <u>GCTCAGGAGAGATTCTAAATTACTACTTGTA</u><br><u>TTTCTTGACTTCTCTTAAACCAACAATG</u> 3' | <u>6239-6328</u><br><u>5627-5655</u> |
| 9           |             | 5' AAGATCACATTGTTGGTTTTAAGAGAAGTCAAGA<br><u>AATTACAAGTAGTAATTTAGGAATCTC</u> 3'      | 5627-5662<br><u>6301-6328</u>        |
| 10*         | <i>NotI</i> | 5' GGGTAT <b>GCGGCCGC</b> ATTTAGGTGACACTATAGA<br>ATACGCAAATTGCGTATAGTATATAAAT 3'    | 1-26                                 |

\* Primers taken from Benjeddou *et al.* 2002

Restriction sites in bold, SP6 promoter in italics and overlapping regions underlined

#### 5.3.4 Reverse transcription of viral RNA and amplification of genome

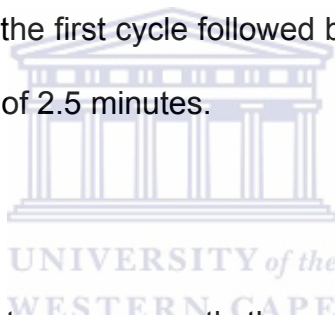
The extracted RNA was reversed transcribed using the Universal RiboClone cDNA synthesis system (Promega) with primer 2 for BQCV and primer 1 for APBV to synthesise full length single stranded (ss) cDNA. The reaction was carried out as described by Benjeddou *et al.* (2002).

The genome halves 5' BQCV and 3' ABPV were amplified using the Expand 20kb<sup>PLUS</sup> PCR system (Roche Diagnostics) with the PCR being performed in 0.2ml thin wall PCR tubes using a Perkin Elmer 9600 thermocycler.

The sscDNA from the reverse transcription (RT) reaction was used directly without purification as the template. The 5' half of the BQCV genome was completed in a PCR reaction with a total volume of 50  $\mu$ l. Each reaction contained 2  $\mu$ l template, 0.5 mM of each deoxynucleotide triphosphate, 0.3  $\mu$ M forward primer 10 and 0.3  $\mu$ M reverse primer 3, 5  $\mu$ l 10 X PCR buffer and 0.75 mM MgCl<sub>2</sub>. The 3' half of the ABPV genome was also completed in the same way as for the 5' half with forward and reverse primers 4 and 6. The PCR profile for the 5' half of BQCV was started with an initial denaturation stage at 94°C for 2 min followed by one cycle of 93°C for 15s, 59°C for 30s and 68°C for 6 min. The following 29 cycles were at 92°C for 10s, 71°C for 30s, 68°C for 6 min with the final elongation performed at 68°C for 10 min. The profile for the 3' ABPV half was identical to that of the 5' half except that the annealing temperature was 61°C in the first cycle and 70°C in the following 29 cycles with elongation times of 3 min. Both of the PCR products were gel purified using the High Pure PCR

purification kit (Roche Diagnostics) to ensure that the DNA had been purified and no carry over contamination occurred in the following steps.

The PCR for the opposite orientation of the chimeric virus was identical to the above except with the 5' ABPV genome half being amplified with primers 7 and 9 and the 3' BQCV genome half with primers 4 and 5. The same reaction volumes and concentration in the reaction mixture was used for the 5' BQCV and 3' ABPV halves as mentioned previously. The cycle temperatures were identical except that the annealing temperature for 5' ABPV was 59°C for 6 min in the first cycle and 71°C in the following 29 cycles. For the 3' BQCV PCR the annealing temperatures were 61°C for the first cycle followed by 70°C in the subsequent 29 cycles with elongation times of 2.5 minutes.



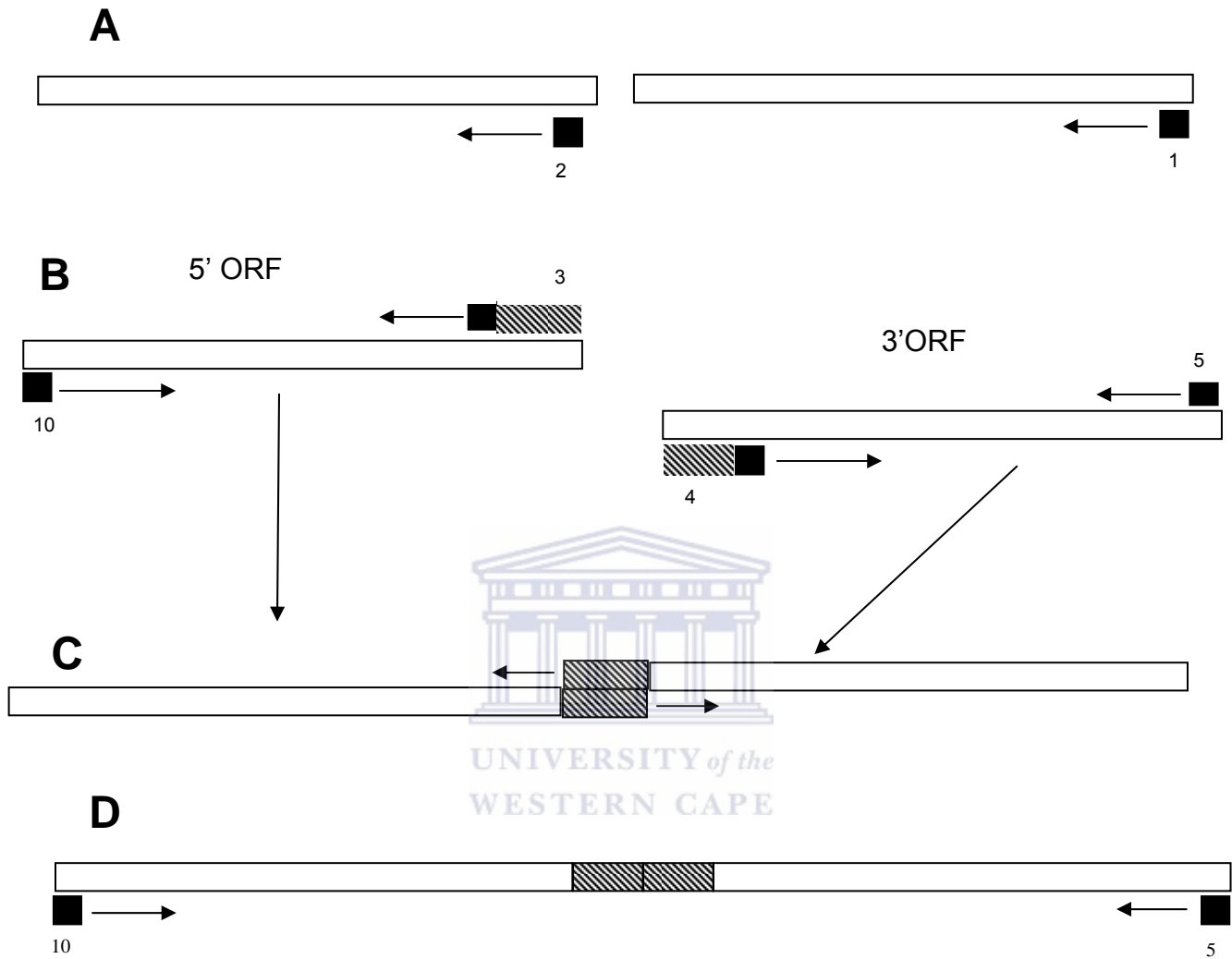
### **5.3.5 Fusion PCR**

The fusion PCR reaction mixture was exactly the same as for the amplification of the two genome halves except that a mixture of DNA starting at 70 ng of the 5' BQCV half and 50 ng of the ABPV half was used as template and the same concentrations were used for the opposite orientation. The reaction mix excluding the primers were heated to 94°C for 2 min after which primer number 10 and 5 for the BQCV/ABPV chimera or 7 and 6 for the ABPV/BQCV chimera was added. One cycle of 92°C for 10s and 80°C for 30s and 68°C for 9 min was used to extend the templates over the entire genome. The primers were then added at 92°C at the beginning of the third stage, which consisted of 29 cycles of

92°C for 10s, 70°C for 30s and 68°C for 9 min. The final extension was performed at 68°C for 20 min.



Reverse transcription and fusion PCR strategy for 5' BQCV and 3' ABPV orientation



**Figure 5.1**

Polymerase chain reaction strategy for the development of the honeybee chimeric virus, adapted from Gritsun and Gould (1995) and Benjeddou *et al.* (2002). The 5' genome half of BQCV and the 3' genome half of ABPV both honeybee viruses were used for construction of the chimera. A. represents the first step of reverse transcription of the RNA to synthesise full length BQCV and ABPV using extracted RNA to produce full length sscDNA. B. represents the amplification of the 5' half of BQCV and 3' half of ABPV with overlaps introduced by the primers represented by hashed squares. C. represents the fusion PCR using the genome halves with the overlapping areas acting as primers in the first cycle of the PCR and finally, D, the full fusion product being amplified using primers 10 and 5 in following cycles to produce the BQCV/ABPV chimeric virus.

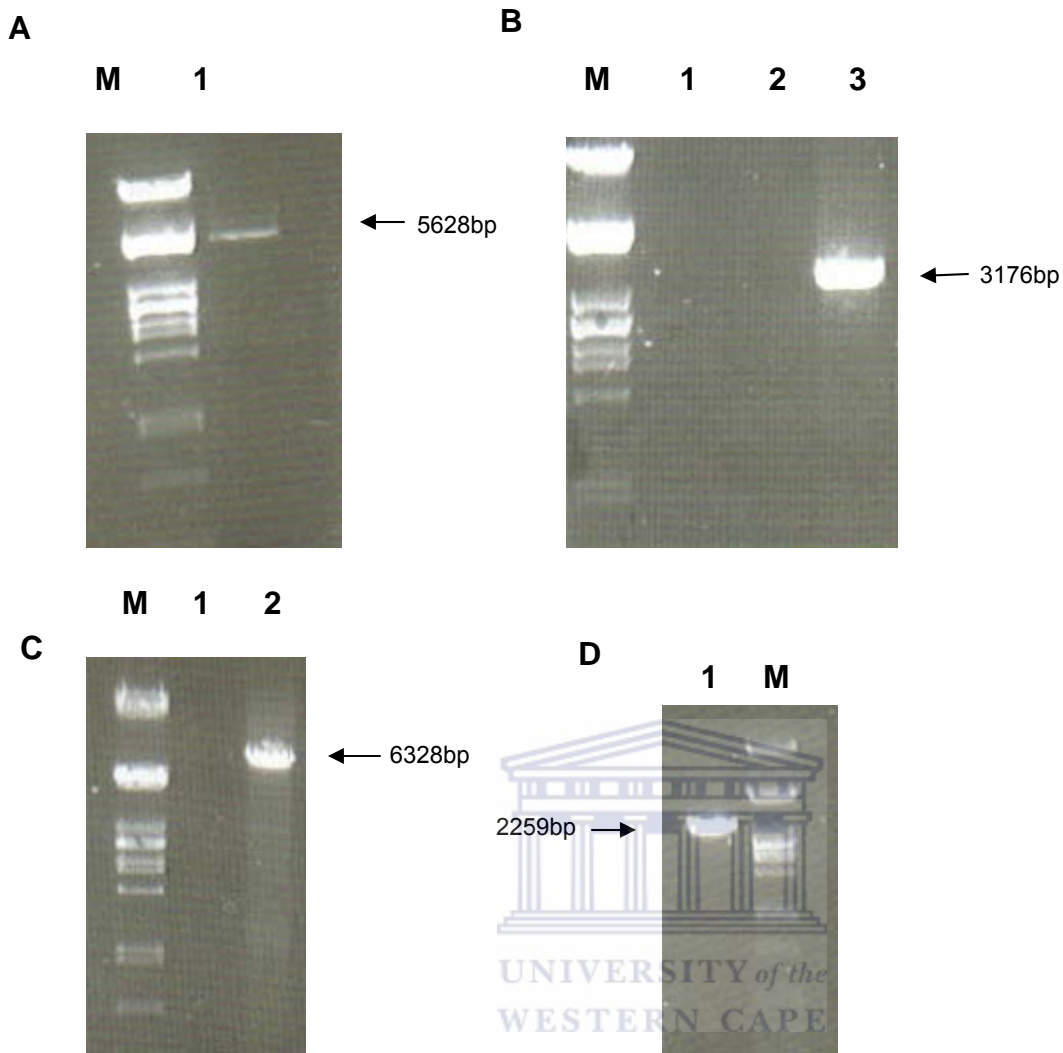
## 5.4 Results

The strategy followed to produce the chimeric virus was one used by Gritsun & Gould (1995) to produce infectious viruses and also used by Benjeddou *et al.* (2002). See figure 5.1 for a diagram of the strategy used to attempt the chimeric honeybee virus.

The primers used in this study were designed from the BQCV and ABPV sequence data entered on the NCBI database. The primers amplified the entire ORF1 including the stop codon when the 5' half was amplified. This ensured that the entire 5' ORF encoding the non structural genes would be amplified from both viruses. Primers for the 3' ORF were designed to include the intergenic region (IGR) between the 5' and 3' ORF of the virus when amplifying the 3' half of the genome. This would ensure the entire 3'ORF including the IGR of the second virus would be present when the two components were to be fused. Therefore the full ORF of each virus would be present and be potentially functional if any internal ribosome entry sites were present. The primers for the PCR strategy were also designed so that an overlapping area of 30 bp were present complementary to the other virus within the IGR. The 30 bp overlap would act as the primer in the first cycle of the fusion PCR demonstrated by Gritsun & Gould (1995) and Benjeddou *et al.* (2002).

The full length genomes of both the BQCV and ABPV genomes were synthesised by reverse transcription using the RNA of each virus and the corresponding primers BQCV primer 2 and ABPV primer 1 as detailed in the

materials and methods section. The sscDNA was then directly used for the amplification of the 5' genome half of BQCV and the 3' genome half of ABPV with the corresponding primers (Fig 5.2). The two amplicons of the genomes halves were then used at different DNA concentration ratios in a fusion PCR with the correct primers. The fusion PCR was attempted with differing DNA concentration ratios between the 5' and 3' halves due to the template size differences. The differences in amplicon sizes would cause favouring during the PCR reactions with primer and template competition to obtain the full length product. The fusion PCR product of the chimeric virus which would contain the 5' half of BQCV and 3' half of ABPV was unsuccessful at every attempt. After these attempts primers were designed to amplify the 5' genome half of ABPV and the 3' half of BQCV to determine if the chimera would be successful in this orientation. In this orientation the two genome halves were once again amplified with the correct primers. Different DNA template concentration ranges were also implemented, however the fusion PCR product again failed to amplify the full chimeric virus of ABPV and BQCV.



**Figure 5.2**

PCR amplification of the genomes halves from single stranded (ss) cDNA. The specific primers produced overlaps to enable the subsequent fusion PCR to take place. Genome halves were all successfully amplified from sscDNA at the appropriate amplification sizes. A, lane 1, 5' BQCV, B lane1 and 2 negative controls, lane 3, 3' ABPV, C, lane 1 negative control, lane 2, 5' ABPV, D, lane 1, 3'BQCV. Lane M in A, B, C and D Lambda *Pst* molecular weight marker.

## 5.5 Discussion

With the development recently of a reverse genetics system for BQCV which showed the ease of manipulation of this genome (Benjeddou *et al.*, 2002) a unique opportunity arose to use the same strategy to investigate the potential of producing a chimeric virus with viral components of BQCV and another honeybee virus ABPV. The correctly fused chimeric virus containing BQCV and ABPV viral components could be useful to observe whether the chimeras as one complete virus would be functional and or infectious. It would also be used to observe the compatibility between structural and non structural genes of the two viruses. Previously no other research has attempted to fuse different ORFs from two honeybee viruses to study the effect or possibility of a chimeric honeybee virus.

Few other chimeric viruses have been produced using fusion PCR, however in the case of swine vesicular virus (Dekker *et al.*, 2000) a similar strategy was followed to produce eight chimeric viruses by substituting different regions of the P1 region of the parent strains with that of different isolates of this virus. Other strategies used unique restriction sites either already present in the genome or introduced to facilitate the joining of the substituted parts of a virus (Kuhn *et al.*, 1996, Pletnev & Men, 1998). Chimeric virus constructs are usually between viruses in the same family or different strains of the same virus (Dekker *et al.*, 2000).

The failure to obtain chimeric viruses in either orientation of the proposed chimeric virus could be due to various factors. One such factor could be the primers designed which introduced the overlapping base pairs to accommodate the fusion PCR. The number of nucleotides in these primers totalled 60 bp which could have impacted on the PCR reactions where the primers, especially those introducing the overlap, could have been too long or too short. Dekker *et al.* (2000) used overlapping regions of 75-90 bp excluding the specific primers in their fusion PCR. Yao *et al.* (1996) only used a 14 bp overlap however it was the same gene in a different alphavirus being substituted and was highly homologous. Benjeddou *et al.* (2002) only used a 6 bp overlap which introduced a mutation in the same virus. Therefore the length of the overlaps can vary and optimising the area in which the overlap was designed and length could possibly directly impact the PCR amplification.

Another factor which could have impacted on the amplification was that the 5' and 3' genomes halves were approximately 6 Kb and 3 Kb respectively. This difference in size could not be overcome by differing the DNA concentration ratios of the genome halves as the optimum ratio between the two halves could not be found. Benjeddou *et al.* 2002 successfully used a ratio of 50:45 ng of the 5' half and 3' half respectively, however the DNA fragment sizes were not as vast as in this study. Gritsun & Gould (1995) also used a ratio of 30 to 50 ng of DNA, however once again the sizes of the two halves were almost identical where the 5' half was 500 base pairs longer than the 3' half.

The joining of two DNA strands of varying lengths has been demonstrated (Shevchuk *et al.*, 2004) however in this case the fusion of the two halves was not possible. The strategy used to produce the chimera will therefore have to be adapted. A strategy more widely used to produce chimeric viruses can also be looked at.

Another possibility would be to use two viruses which are more closely related such as ABPV and KBV. At the outset of the study BQCV was chosen since previous work had obtained a successful infectious virus with fusion PCR.

Although the chimeric fusion PCR was not possible, with new possibilities of using multiple gene cloning baculovirus vectors and cell culture could improve the chances of creating chimeric honeybee viruses. This could be achieved by cloning the structural and non structural genome halves of the virus and expressing it at the same time in a multiple expression vector in cell culture and observing if fully functional virus particles are being produced. Having a cell culture system has many advantages for the further study and characterisation of honeybee viruses in the future.

## CHAPTER SIX

### CONCLUSIONS

As emphasised with this work honeybees are very important insects not only environmentally but economically as well. With concerns growing everyday about honeybee colony collapse disorders and factors such as climate change influencing honeybee populations worldwide a dire need for research has been acknowledged. Research and developing new methods to combat disease and colony collapse due to pests should get the attention it deserves in order to protect the honeybee. Molecular techniques, as used in this work, has greatly increased the knowledge about honeybee viruses and will continue to do so with on going research.

The detection of three honeybee viruses is an attempt to shorten the time needed to diagnose viruses within a colony or hive. With more viruses being found and their genomes sequenced primers can be designed in order to detect these viruses by PCR methods. A multiplex PCR is unique in that many viruses can be detected simultaneously in one reaction. Therefore with more research this method has the potential to detect many different viruses within a colony.

Sequencing of the honeybee virus genomes has assisted with methods for detection of the viruses. KBV is a virus which has caused high mortality in many countries. With the sequencing of a strain of KBV from North America it could be used to compare a KBV virus strain from India. The KBV Indian strain was used in the multiplex RT-PCR however was removed due to primer interactions. The KBV strains were seen to differ in the region looked at due possibly to

geographical strain differences. The KBV Indian strain was however highly homologous to ABPV within the region of the primers being designed. Therefore correct design and alignments are crucial to ensure that this detection method is used to its full potential.

Heterologous baculovirus expression of the BQCV capsid gene in insect cell lines was shown. The morphology of the viral capsid was similar to that of the wildtype virus as seen by electron microscopy of the expressed VLPs. This expression system has the potential to be used to express the entire BQCV virus. Traditional propagation methods are tedious therefore a cell culture method to propagate honeybee viruses would be an improvement. Cell culture propagation would ensure that the virus being propagated was pure and that any inapparent or contaminating viruses would be excluded. With many commercial vectors available for multiple gene cloning capabilities using heterologous baculovirus expression, with further study, can be used in the near future for the propagation of honeybee viruses. The studies which could be conducted with these systems and viruses may possibly be used to elucidate how these viruses work and infect. Chimeric viruses have been used in many studies to observe the infectivity and virulence of these altered viruses. Developing chimeric viruses between highly virulent and disease causing viruses of the same family have led to candidate vaccine production and attenuating the virulence of the viruses. The same can be done for honeybee viruses which are causing huge losses within this industry. If an attenuated honeybee virus could be developed this would attempt to find a possible vaccine for the diseases. A chimeric honeybee virus was attempted

between ABPV and BQCV however the strategy followed failed to fuse the two components of the virus. Further optimisation of the study and using methods such as heterologous expression in baculovirus culture could see the development of a chimeric virus. The resultant virus could be used in many studies in which to help researchers find ways of inhibiting viral transfer and infection in honeybees.

Improvements on methods used for research is ongoing and honeybee virus research is no different. Propagating pure viruses with which to conduct studies is important and the methods to do so has to be considered. Looking more closely at these viruses by using new methods of propagation will assist in the research to save the honeybee.



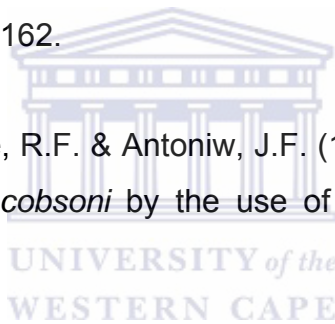
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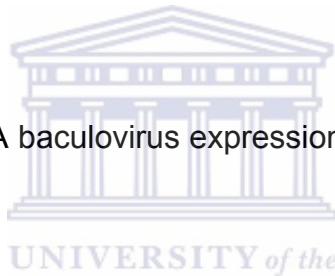
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