Molecular detection and characterisation of RNA viruses of honeybees

By

Elize Lindsay Topley

Submitted in partial fulfilment of the requirements of the degree of

Philosophiae Doctor

In the Department of Biotechnology

University of the Western Cape

Supervisor: Prof. Sean Davison

2009

DECLARATION

I declare that "**Molecular detection and characterisation of RNA viruses of honeybees**" is my own work and that all the resources I have used or quoted have been indicated and acknowledged by means of complete references.

Elize Lindsay Topley

ABSTRACT

Honeybees have evolved through the centuries to inhabit most parts of the world except for the extreme Polar Regions. These insects have also been susceptible to pathogens and disease which has always been part of the honeybees' ecology and has evolved and adapted accordingly. However disease has spread more rapidly into areas where no disease existed before with the transport and moving of hives. Disease has caused massive losses within the honeybee industry in recent history. Using new technology available to scientists, diseases and parasites can be identified and this information used to prevent damage to hives, the livelihood of many crop farmers and beekeepers around the world. Of these diseases honeybee viruses have become of some concern in recent times.

Honeybee viruses' black queen cell virus (BQCV) and acute bee paralysis virus (ABPV) were found to have genomes consisting of 8550 and 9490 nucleotides respectively. The viruses have two open reading frames (ORFs) which encode a non structural protein at the 5' ORF and a structural protein at the 3' ORF. Sacbrood virus (SBV) has a different organisation to BQCV and ABPV where it has a single ORF with the structural genes at the 5' end and the non structural genes at the 3' end. In an effort to rapidly identify honeybee viruses a multiplex reverse transcriptase polymerase chain reaction (RT-PCR) was developed for the simultaneous detection of BQCV, ABPV and SBV in a single reaction. Primers were designed within the 3' open reading frame to amplify fragments of 434bp for SBV, 900bp for ABPV and 316bp for BQCV. RNA was extracted from

laboratory infected and naturally infected samples. The PCR products were sequenced and found to be that of the appropriate virus. The primers were tested on naturally infected samples with SBV and BQCV being detected.

Another well characterised honeybee virus Kashmir bee virus (KBV) was initially added to the multiplex RT-PCR. However inconsistencies with the multiplex PCR led to the sequencing of a 2 kilobase fragment of the KBV Indian (KBV-in) strain. Three overlapping cDNA fragments of KBV were sequenced and aligned with the full length sequence of KBV and a sequenced capsid region of KBV both from North America. Alignment to ABPV was also completed to observe the homology between KBV-in and ABPV. The KBV-in strain was not highly homologous to the North American strains over the region which was sequenced for KBV-in. ABPV was also not highly homologous over the entire 2 Kb region. However over the region where primers were designed for the RT PCR of KBV, ABPV was highly homologous at 80%. This could have led to the inconsistencies when PCR was done. Primer design and correct strain characterisation is needed before primers are designed to detect more than one virus per reaction. Further characterisation and sequencing of this strain is needed in order to make further comparisons.

Propagation methods for honeybee viruses have not changed since these viruses were discovered. There are no suitable cell lines or cell culture techniques available for honeybee viruses. Honeybee viruses have to be manually injected with virus in order for the virus to multiply and be extracted. With the presence of inapparent viruses which could co-infect pupae, a method

for pure virus propagations needs to be found. Recombinant baculovirus systems have been used extensively to produce foreign proteins from different viruses using vectors and recombinant technology. In this chapter we inserted the capsid gene from BQCV into a transfer vector under the control of the p10 promoter of *Autographa californica*. Fractions of the sucrose gradient containing the virus like particles (VLPs) were seen under the electron microscope. A Western blot showed the four capsid proteins at the expected sizes for BQCV capsid. This study therefore has shown that a heterologous system such as baculovirus can be used for virus like particle production.

Infectious virus technology has helped gain insight into how viruses work. Using this technology altering honeybee viruses could be used to observe different functionalities of the viruses. An attempt was made to interchange the open reading frames of ABPV and BQCV to observe any changes in virus assembly and infectivity. A fusion PCR strategy was employed to interchange the 5' and 3' ORFs of APBV and BQCV. The strategy however was unsuccessful. Alternative strategies could improve the chances of obtaining a chimeric virus.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the following persons:

Prof Sean Davison, for all the guidance, encouragement and positivity throughout my studies;

Dr Mongi Benjeddou, for all your scientific input and constructive criticism over the years;

Prof Sehaam Khan, Michael Tobin and Rayaana Abrahams-Fredricks, for all their time spend answering questions, providing the baculovirus cell cultures and helping with all the protein gels completed in chapter 4. Thank you;

SunBio colleagues at Stellenbosch University for your encouragement and patience over the last four years;

National Research Foundation for financial support.

Finally, thank you to my family for their love, support and patience over the years while I completed my studies. Thank you.

CONTENTS		PAGE NO.

LIST OF FIGURES	PAGE NO.

LIST OF TABLES **PAGE NO.**

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

The history of honeybees dates back many centuries with the European honeybee (*Apis mellifera* L.) been extensively populated in many areas of the world by settlers. Honeybees were introduced to the new world specifically America, Australia, and Siberia roughly in the 16th century (Dietz, 1992). Today many centuries later beekeeping has grown into an economically important business for not only are honey products such as honey, wax, royal jelly, bee venom and pollen sold but hives are used in crop fields to pollinate various flowering crops (Johannesmeier & Mostert, 2001). Honeybees visit millions of flowers in their lifespan gathering pollen, subsequently and inadvertently pollinating flowers as they go along.

It has been estimated that bees pollinate 60-70% of all flowering plants in South Africa surpassing the economical monetary value of honey products (Johannesmeier & Mostert, 2001). The ecological advantages of honeybees pollinating wild flowers, garden plants and other plant and tree species, helps to maintain the biodiversity of these wild or indigenous flowers. This in turn provides a food source for many birds and small animals (Williams, 1994). Also the pollination of important commercial crops leads to the increase in yield of the crop, fruit size and increases the earliness and uniformity of seed sets in crops.

The value of the commercial crops pollinated by honeybees has been estimated at 2.8 billion Rands per annum (Johannesmeier & Mostert, 2001).

In South Africa the detection of pests and disease in apiaries has put many hives under increasing pressure due to a decrease in viability. Two species of mites can be found in South Africa namely the tracheal mite (*Acarapis woodii*) and the varroa mite (*Varroa destructor*) (Buys, 1995; Allsopp *et al.,* 1997). The varroa mite in particular has been of concern in recent years in many parts of the world and increasingly in North America. This mite has caused and said to be responsible for the loss of a large number of colonies (Kraus & Page, 1995; Finley *et al.,* 1996). Suggestive evidence has been found that the colony losses due to the mite are in actual fact an association between honeybee viruses and the varroa mite rather than the mite acting by itself (Bailey *et al.,* 1983; Ball & Allen, 1988; Allen & Ball, 1996; Brødsgaard *et al.,* 2000). Recently a new threat to honeybees has surfaced in North America with entire hives dying. The causative agents of the mortality of the hives are not known and the syndrome has been called colony collapse disorder (Cox-Foster *et al.,* 2007).

1.2 Honeybee parasites

1.2.1 Varroa mites

The parasite *Varroa destructor* (formerly *jacobsoni*), the most economically important parasite of honeybees, was discovered in the hives of the Asian bee *Apis cerana* from Java (Oudemans, 1904). *V. destructor* was then reported for the first time to have infested *Apis mellifera* in 1962 (Delfinado, 1963). The mite,

known as *V. jacobsoni* Oudamans, was taxonomically changed to *Varroa destructor* (Anderson & Trueman, 2000). This change was made when studies carried out on the genetic variation of varroa lead to the observation that two out of eighteen genetically different mite populations have changed hosts to infest *Apis mellifera* and spread through bee populations worldwide (Anderson, 2000; Anderson & Trueman, 2000). The *V. destructor* mites which infest *Apis mellifera* has spread over the last 50 years from eastern Asia throughout the world causing the mortality of millions of colonies (Anderson, 2000). The spread of varroa is suggested to have taken place by commercial transport of bees and queens, the migratory activities of beekeepers, swarms that fly long distances, ships carrying hives and drifting bees (Swart *et al.,* 2001).

The female varroa mite is a flat oval shaped 1.1mm long and 1.5mm wide pale brown to reddish brown mite. It attaches itself to the adult bee between body regions making them difficult to detect. These areas that they attach to are where the mites have easy access to the hemolymph, which they feed on reducing the bees' life expectancy (De Jong & De Jong, 1983). The mite also parasitises most severely on older larvae and pupae but preferring drone brood to worker brood because of the longer sealed stage (Ritter & Ruttner, 1980).

Infestations of varroa has proven to be quiet difficult to control and eradicate in South Africa especially in populations in the wild that cannot easily be reached (Allsopp, 1997; Swart *et al.,* 2001). In Europe and America some form of varroa treatment is employed due to the huge losses in honeybee colonies in the country caused by varroatosis (disease caused by varroa) (Allsopp, 1997).

However varroa is not the sole cause of colony collapse, in instances where colonies are heavily infested with mites, the colony is further weakened by other diseases and pests including viruses (Swart *et al.,* 2001). It is then these complementary infections and disease that could cause total collapse of colonies The term “bee parasitic mite syndrome” has been used to describe the colony losses due to varroa and virus infection (Shimanuki *et al.,* 1994).

1.2.2 Bee parasitic mite syndrome

The term “bee parasitic mite syndrome” is used to describe the disease complex in colonies which are simultaneously infected with virus and infested with mites and leads to the collapse of the colony (Shimanuki *et al.,* 1994). *Varroa destructor* has been suggested to either act as an activator of inapparent viruses or as a vector for the virus (Ball & Allen, 1988; Bowen Walker *et al.,* 1999) but still their relationship is not fully understood. The suggestion that mites vectored the virus was supported by the detection of a virus in the mite by indirect ELISA techniques (Allen *et al.,* 1986). Bowen-Walker *et al.* (1999) detected deformed wing virus (DWV) in mite populations infesting colonies succumbing to DWV infection and recently acute bee paralysis virus (ABPV) was detected in mite samples using reverse transcriptase–PCR techniques (Bakonyi *et al.,* 2002). Their results showed that ABPV could be detected in mites but not all samples tested contained ABPV. More conflicting results surfaced when records in Belize and Nicaragua showed that ABPV was detected in large amounts in dead adult bees where these countries are supposedly mite free (Allen & Ball, 1996).

Experiments carried out by Hung *et al.* (1996) found that colonies with bee parasitic mite syndrome contained dead adult bees that were virus free. This lead to the suggestion that the virus and the mite are part of a complex problem that caused the mite infested colonies to collapse (Hung *et al.,* 1996; Martin, 2001).

1.3 Honeybee viruses

1.3.1 Virus classification

There are approximately eighteen honeybee viruses that have been identified throughout the world (Ball & Allen, 1988; Allen & Ball, 1996). The viruses are morphologically indistinguishable from each other when studied under an electron microscope. Field diagnosis is unreliable because not all viruses exhibit visible symptoms. Many of the viruses consist of 30nm isometrical particles and are all positive stranded RNA viruses except for one, which is a DNA virus. The viruses where discovered in various parts of the world whether parasitic mites were present or not. The complete genome sequences of eight viruses have been published namely acute bee paralysis virus (ABPV), black queen cell virus (BQCV), sacbrood virus (SBV), Kashmir bee virus (KBV), deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), chronic bee paralysis virus (CBPV) and slow paralysis virus (SPV). These viruses and others are characterised according to physical features and classification in Table 1.1.

Table 1.1 Classification of viruses isolated from honeybees which have been sequenced.

Name	Genome	Family	Accession no.	Reference
Acute bee paralysis virus	RNA	Dicistroviridae	NC 002548	Govan *et al.,* 2000
Black queen cell virus	RNA	Dicistroviridae	NC 003784	Leat *et al.,* 2000
Kashmir bee virus	RNA	Dicistroviridae	NC 004807	de Miranda *et al.,* 2004
Deformed wing virus	RNA	Iflaviridae	NC 004830	Lanzi *et al.,* 2006
Israel acute paralysis virus	RNA	Dicistroviridae	NC 009025	Maori *et al.,* 2007
Sacbrood virus	RNA	Iflaviridae	NC 002066	Ghosh *et al.,* 1999
Slow paralysis virus	RNA	Iflaviridae	EU35616	Unpublished
Chronic bee paralysis virus	RNA	Unclassified	EU122229/ EU122230	Olivier *et al.,* 2008

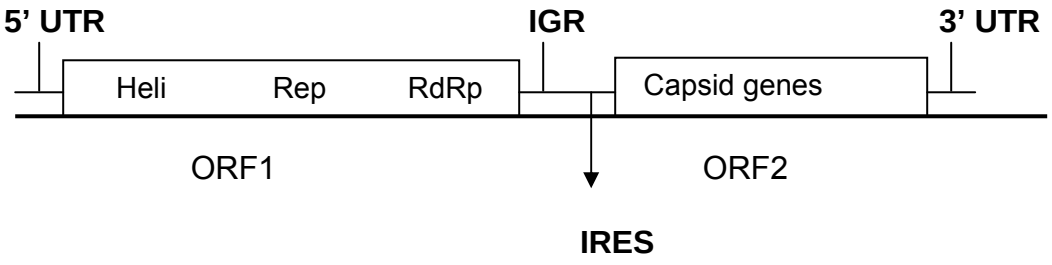
1.3.2 Viral genome organisation

Honeybee viruses resemble picornaviruses as do many other insect infecting RNA viruses. The molecular and phylogenetic studies completed on the sequenced honeybee viruses ABPV, BQCV, SBV, KBV, DWV and other viruses associated with honeybees like varroa destructor virus 1 (VDV 1) has shown that they do not belong to the picornavirus family but are picorna-like in many ways (Govan *et al.,* 2000; Leat *et al.,* 2000; Ghosh *et al.,* 2001; de Miranda *et al.,* 2004; Lanzi *et al.,* 2006; Ongus *et al.,* 2004). SBV, DWV and VDV 1 are monocistronic viruses in this group and are said to resemble the infectious flacherie virus of silkworms. Since this discovery all insect viruses including these three which have a single open reading frame with structural proteins at

the N terminal side of the virus have been grouped as *Iflavirus* (Christian *et al.,* 2002). BQCV, ABPV and KBV and all other insect bicistronic viruses belong to a novel group of insect viruses, formally the cricket paralysis like viruses, and recently renamed family *Dicistroviridae*, genus *Cripavirus*. The *Discistroviridae* group consists of infecting RNA viruses such as cricket paralysis virus (CrPV), *Drosophila* C virus (DCV), *Plautia stali* intestine virus (*PS*IV), *Rhopalosiphum padi* virus (*Rh*PV) and himetobi P virus (Mayo, 2002) and the honeybee viruses ABPV, BQCV and KBV mentioned previously.

The genomes of the bicistronic viruses have non structural genes encoded by the 5' proximal open reading frame (ORF) and structural genes encoded by the 3' proximal ORF. Figure 1.1A depicts the organisation of the genomes of the *Discistroviridae* with the helicase, 3C protease and RNA dependent RNA polymerase (RdRp) followed by the capsid protein, also shown are the 5' and 3' untranslated regions (UTR) and the intergenic region (IGR). In figure 1.1B the genome organisation of Iflavirus is also shown.

(A)



(B)

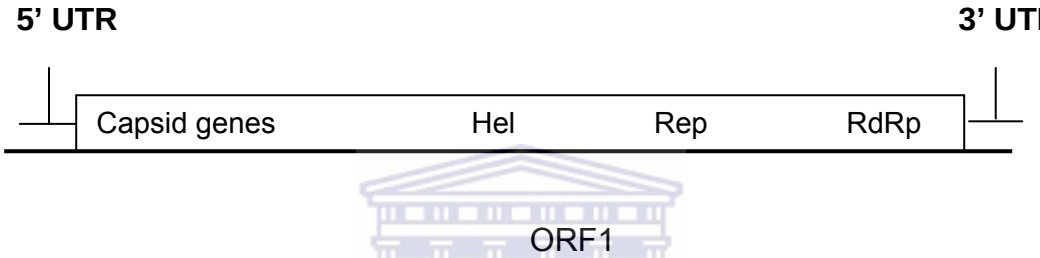


Fig. 1.1

Schematic diagrams of the genome organisation of two virus families which have been found in the honeybee. (A) *Dicistroviridae* organisation. The open boxes represent the open reading frame (ORF) 1 and ORF 2. Indicated in the diagram is the 5' untranslated region (UTR) the intergenic region (IGR) between the two ORF's and the 3' untranslated region (UTR). Also indicated is the suggested area for IRES activity which has been found in several of the viruses in this family. (B) Iflavirus organisation. The open box represents the single open reading frame consistent with viruses in this family. The genome is divided by the structural genes occurring in the 5' region of the genome and the nonstructural in the 3' region of the genome. The 5' and 3' UTR are also indicated.

Previous studies on *Dicistroviridae* viruses have revealed that PSIV, *Rh*PV and CrPV all have internal ribosome entry sites (IRES). Mediated initiation of both the 5' and 3' proteins in *Rh*PV and CrPV by IRES activity have been proven

where as IRES mediated initiation occurs for the 3' proteins in PSIV (Woolaway *et al.,* 2001; Domier *et al.,* 2000; Wilson *et al.,* 2000; Saski & Nakashima, 1999). It was also shown that PSIV and *Rh*PV IRES elements initiated translation at non AUG codons (Domier *et al.,* 2000; Sasaki & Nakashima 1999). It was later also suggested that the 3' proximal ORF of BQCV was facilitated by an IRES element at the CCU codon since no AUG codon appears at the start of the coding region of this virus (Leat *et al.,* 2000).

1.3.3 Honeybee virus, Black queen cell virus

Black queen cell virus (BQCV) was first isolated from queen pre-pupae and pupae found dead in their cells (Bailey & Woods, 1977). The virus name was derived from the darkened areas on the walls of the cells containing the infected pupae. BQCV is often detected when colonies are infested with the microsporidian mite *Nosema apis* (Allen & Ball, 1996; Bailey *et al.,* 1983) and was seen to cause mortality of bees infected with this parasite in some instances. Studies have identified BQCV as having 30nm particles and a positive stranded RNA genome. The South African isolate of BQCV was found to have a genome consisting of 8550 nucleotides excluding the poly (A) tail. The genome consists of two ORF in line with other picorna-like viruses where the 5' proximal ORF encoding a putative replicase protein and the 3' proximal ORF encoding a capsid polyprotein (Leat *et al.,* 2000). Using multiple sequence alignments Leat *et al.* (2000) also identified three domains characteristic to helicase, 3C like cysteine protease and RNA dependent RNA polymerase (RdRp) which lies in the 5'

proximal ORF (see fig 1.1A). The virus contains four capsid proteins with molecular masses of 34, 32, 29 and 6kDa. Evidence was found that certain picorna-like viruses has translation initiation facilitated by IRES elements. It was then suggested that BQCV 3' proximal ORF is facilitated by an IRES at a CCU codon (Leat *et al.,* 2000). The virus was classed as *Dicistroviridae* as were other insect viruses having the same genome organisation (Mayo, 2002).

1.3.4 Acute bee paralysis virus

Acute bee paralysis virus (ABPV) was found originally as an inapparent infection of honeybees (*Apis mellifera*) in Britain (Bailey *et al.,* 1963). ABPV is the only honeybee virus which has a natural alternate host where the virus was also found in infected bumblebees (Bailey & Gibbs, 1964). ABPV spreads by way of the salivary gland secretions of adult bees. The virus can also be found in the food stores, to which the salivary secretions are added (Ball, 1985). ABPV has shown to cause colony loss in both adult bees and larvae when colonies are infested with the mite *Varroa destructor*. The mite has been implicated as acting as a vector for the virus when feeding on the hemolymph of the honeybee (Scott-Dupree & MacCarthy, 1995). The ABPV genome consists of positive sense single strand RNA with the genome consisting of 9470 nucleotides. The virus has two ORFS with the non-structural genes in the 5' ORF and the 3' ORF contains the structural genes. The capsid proteins were identified and the sizes of the four proteins were 35, 33, 24 and 9.5kDa (Govan *et al.,* 2000). The virus

was re-classes as *Dicistroviridae* as it resembles other insect infecting viruses with the same genome organisation (Mayo, 2002).

1.3.5 Kashmir bee virus

Kashmir bee virus (KBV) was discovered in 1974 as a contaminant in a preparation of *Apis* iridescent virus from an *Apis cerana* hive (Bailey & Woods, 1977). Although suspected to have originated in *A. cerana* and South East Asia KBV was also discovered in natural populations of *A. mellifera* from around the world (Ball & Bailey, 1997; Allen & Ball, 1995). Previous studies have suggested that KBV is closely related to ABPV both serologically and biologically (Allen & Ball, 1995; Anderson, 1991). However evidence was found that ABPV and KBV differ in key areas across the genome of the viruses (de Mirande *et al.,* 2004). KBV has also been implicated as being an inapparent virus and can multiply to high titres when extracts of apparently "healthy" bees were injected or fed to larvae and bees (Dall 1985). The varroa mite has also been suggested to vector the virus in colonies infested with this mite (Bailey & Woods, 1977). KBV consists of positive sense RNA genome containing 9524 nucleotides (de Miranda *et al.,* 2004).

1.3.6 Sacbrood virus

Sacbrood virus was characterised in 1964 in honeybees. It has been observed that SBV is the most common viral disease of bees found on every continent (Dall, 1985; Nixon 1982). The brood of the honeybee is usually infected by SBV

resulting in the death of the larvae. Larvae Infected with the virus fail to pupate and ecdysial fluid rich in SBV can be found accumulated under the unshed cuticle forming the sac (Shen *et al.,* 2005). SBV may also infect adult bees without any obvious signs of disease (Allen and Ball, 1996). The nucleotide sequence of SBV is available and unlike ABPV, BQCV and KBV falls within the Iflavirus family as described in the previous section (Ghosh *et al.,* 1999).

1.4 Virus prevalence

The distribution of viruses throughout the world has been well documented, however due to the special techniques needed for virus detection the results could be incomplete. Many honeybee viruses persist as inapparent or latent viruses which lie dormant until activated causing the viruses to multiply (Allen & Ball, 1996). Most honeybee viruses have no overt symptoms that can be observed by eye and detection can be time consuming. Only SBV and CBPV can be readily diagnosed by symptoms when these viruses occur naturally in honeybee colonies. Therefore the diagnosis of honeybee viruses can rarely be diagnosed by symptoms or electron microscopy, due to most honeybee viruses being morphologically indistinguishable. There has also been evidence that two or more honeybee viruses can be present in the same hive at the same time and in some instances in the same bee (Evans, 2001; Chen *et al.,* 2004; Topley *et al.,* 2005). Many studies carried out on virus distribution had been initiated due to the viruses association with the varroa mite (Ball, 1993). The viruses which persist as inapparent infection often have very low viral particle numbers that

cannot be readily detected. These viruses however can be easily induced by injecting bees or pupae with extracts from apparently healthy bees from the same colony which leads to the viral particles multiplying to detectable levels (Anderson & Gibbs 1988; Allen & Ball, 1996). The detection of these viruses can be carried out by various immunodiffusion tests or molecular techniques.

1.5 Detection of viruses

There are several methods used for the detection of honeybee viruses. These include enzyme linked immunosorbent assay (ELISA) immunodiffusion, enhanced chemiluminescent western blotting and reverse transcriptase (RT) PCR (Allen & Ball, 1996; Allen *et al.,* 1986; Stoltz *et al.,* 1995) and recently a real time quantitative RT-PCR for detection of viruses (Chantawannakul *et al.,* 2006; Chen *et al.,* 2005). Still widely used today the immunodiffusion technique is rapid, inexpensive and specific (Allen & Ball, 1996).

1.5.1 Serological methods

The most widely used serological method for the identification of honeybee viruses is the ELISA test. This entails raising antibodies to the different honeybee viruses which have been discovered employing the ELISA method for identification. Briefly the ELISA method uses the quantitative estimation of the amount of virus present in a sample relevant to the colour reaction produced by the assay when the antibodies react with the virus (Ball, 1997). However, serological methods have proven to be unreliable in certain instances where

these methods depend on precise antibodies being developed for each member of a broad family of honeybee viruses. The antibody stock also has to remain current and inclusive of all possible viruses. This continuous development of antibodies does seem unlikely to occur for honeybees (Evans & Hung, 2000). Another drawback of serological assays are the cross reactivity with other viruses, which has been shown to co-infect a single bee (Evans, 2001).

In another serological method used, immunodiffusion uses agar jelly layers where the bee extracts are placed. The virus antiserum is also placed on the jelly and both virus extracts and antisera migrate through the agar. An insoluble complex forms or visible line when specific antisera and viruses meet in the agar (Ball, 1997). Immunodiffusion tests are relatively insensitive because a very high concentration of virus is required which is not normally found in bees where inapparent infections are present. The test is useful though when testing the cause of mortality in natural disease outbreaks (Ball, 1997).

1.5.2 Molecular methods

The use of molecular methods for detecting and identifying honeybee viruses is fairly common and genome sequences of honeybee viruses are being deposited more frequently into databases. The use of molecular techniques such as polymerase chain reaction (PCR) reverse transcriptase PCR (RT-PCR), multiplex RT-PCR and real time PCR has become more widely used. The RT-PCR technique has been used to detect KBV (Stoltz *et al.,* 1995), BQCV and ABPV (Benjeddou *et al.,* 2001) and SBV (Grabensteiner *et al.,* 2000). The RT-PCR has

proven to be reliable, specific and sensitive for the identification of honeybee viruses from field samples where virus titres are usually low. Molecular methods require only a few nano grams of viral RNA in order to identify the virus. A multiplex RT PCR system for the simultaneous detection of three or more viruses put into a reaction has also been developed. The detection of three important viruses ABPV, BQCV and SBV in a single reaction from laboratory infected and naturally infected bees was observed to be sensitive in detecting these honeybee viruses (Topley *et al.,* 2005). More recently a real time quantitative RT-PCR method was developed which was able to detect multiple viruses in bees and mite samples and also quantify all the viruses present in one reaction (Chantawannakul *et al.,* 2006; Chen *et al.,* 2005).

1.6 Alternative systems for virus propagation and expression

Honeybee virus propagation has remained a time consuming task where honeybee pupae or adults have to be manually injected or fed virus particles (Bailey & Woods 1974, 1977). These methods have been used since honeybee viruses were discovered and to date no cell culture method has been developed. The short comings of the manual infections include needles used for injecting virus could be contaminated or cause the activation of the virus in the case of inapparent viruses (Anderson & Gibbs 1988). Therefore the injected virus as well as the inapparent virus will be propagated leading to a mixed infection and not a pure virus strain of the desired injected virus. Mixed infections have occurred naturally where two or more viruses are known to inhabit the same bee

(Evans and Hung, 2000; Chen *et al.,* 2004; Topley *et al.,* 2005). For further studies to be conducted on honeybee viruses the ability to harvest pure virus is becoming crucial to particularly molecular studies. Pure virus for serological detection and genome or DNA manipulations are important as to inhibit any contamination from another virus. Various technologies are available which can be looked at.

There are many heterologous systems which could be considered in order to determine if cell culture for honeybee viruses are viable. These include yeast systems, baculovirus systems and *Drosophila* systems. Baculovirus vector systems and promoters especially have proven very reliable across many different viruses and all of these systems are commercially available.

1.6.1 Recombinant baculovirus vector systems

Baculoviruses are one of the largest insect virus groups known which contain double stranded DNA genomes and can reach in excess of 150kb in size. There are two subgroups of baculoviruses with subgroup A being the nuclear polyhedrosis virus (NPV) and subgroup B granulosis viruses (GV). Many different baculovirus genomes have been sequenced of which *Autographa californica* NPV (AcNPV) is one of the most studied (Beljelarskaya, 2001). The host ranges of baculoviruses are mostly *Diptera*, *Hymenoptera* and *Lepidoptera* (Rohrmann, 2008).

Baculovirus expression vectors have become more widely used due to the limitations of bacterial and yeast systems (Kitts and Possee, 1993). The insect

cell lines used for baculovirus expression have many of the post translational modifications known to occur in mammalian systems. These include N- and O-linked glycosylation, phosphorylation, acylation, proteolysis and amidation which occur to some extent in insect cell lines (Hegedus *et al.,* 1997). Recombinant baculovirus technology makes use of a transfer vector which contains all the necessary sequence including promoters, ampicillin resistance and sequence for homologous recombination between the vector and linearised baculovirus. The promoters which are frequently used in many different studies include polyhedron (Ph) and P10 which are very strong constitutive promoters in baculoviruses (Davies, 1995). Another promoter widely used is the immediate early (IE) group of promoters.

The construction of the recombinant baculoviruses in early studies went through many changes to find the best suited method for recombining the transfer vector to the full length baculovirus. Several procedures were proposed including direct enzymatic ligation of the transfer vector into the viral genome which was seen to be very difficult to carry out due to the baculoviruses large genomes (Davies, 1994). This method was not widely used with shuttle vectors and large plasmids also being proposed but these methods were also problematic and again not widely used. The most common methods of recombination between the transfer vector and the virus were by homologous recombination in cell culture. The process involves the wild type baculovirus being linearised and co-transfecting the linear virus with the transfer vector in cell culture (Beljelarskaya, 2001). The sequence flanking the cleavage sites are repaired and the DNA is circularised

again (Kitts *et al.,* 1990). Recombination of the virus and transfer vector for the gene expression depends on two crucial aspects, one being the method of recombination in insect cells and secondly the cell culture conditions. There are two main cell lines used widely, *Spodoptera frugiperda* (*Sf)* including *Sf* 9 and *Sf* 21 cells and *Trichoplusia ni*. Other valuable tools which have also been added to the recombinant vector systems are the selective markers which make selection of recombinant virus plaques easier than time consuming processes of the past. Selective markers used widely are the *E. coli* β-galactosidase gene, controlled by a viral promoter, which in the presence of X gal produce blue plaques which indicate the recombinant viruses with the *Lac Z* altered by the insertion of the foreign DNA fragment (Hegedus *et al.,* 1998). Another marker used for blue/white selection is β-glucuronidase identified in the presence of X gluc (Lerch & Friesen, 1993). The green fluorescent protein (GFP) gene from *Aquorea victoria* is also widely used. The expression of GFP fused with the expressed protein can easily identify recombinant plaques under UV light (Wilson *et al.,* 1997). These are a few of the more widely used selective markers which are available commercially.

1.6.1.1 Expression of virus like particles

The baculovirus expression vector systems have been used widely for the expression of a variety of recombinant proteins in insect cell lines. The proteins produced include cytosolic, nuclear, mitochondrial, membrane bound and secreted proteins. The expression of virus like particles (VLPs) by baculovirus

expression vectors have been utilised increasingly. The VLPs can be used to study viral assembly processes without having an infectious virus and VLPs can also be used for the safe production of vaccines (Kost & Conreay, 1999).

Examples of expressing VLPs by baculovirus vector systems are extensive and cross many different virus families that do not have cell lines available or use insect cell lines due to its ease. The capsid assembly of herpes simplex virus type 1 was investigated by recombinant baculovirus expression (Newcomb *et al.,* 1996). Assembly of polyomavirus was studied using combinations of recombinant baculovirus that encoded VP1, 2 and 3. Where VP1 alone produced VLPs and VP2 and VP3 alone did not (Ke *et al.,* 1999). Vaccines have also been produced using expressed VLPs. Vaccination with purified rotavirus VLPs resulted in protection against a rotavirus challenge (Crawford, 1999).

Recombinant baculovirus expression vectors can be used successfully to express VLPs of many different viruses. These studies have elucidated many different characteristics of the necessary components for capsid assembly. Much can still be learned about viruses using these systems.

1.7 Molecular manipulation of honeybee viruses

1.7.1 Reverse genetics technology for RNA viruses

Analysis and modification of DNA has allowed insight into the organisation and expression systems of the organism being studied. Following DNA modifications,

RNA viruses because of their small size make them perfect volunteers for recombinant technology (Boyer & Haenni, 1994).

Infectious virus clone technology is a modern and direct approach for the analysis and modification of virus genomes at the molecular level (Gritsun & Gould, 1995). The infectious clones are useful in studies which focus on genetic expression, replication, functioning of viral proteins and recombination of RNA viruses. The development of new viral vectors and vaccines is another useful implementation of this technology (Meulenberg *et al.,* 1998; Boyer & Haenni, 1994).

Using this technology infectious viruses usually start out as reverse transcribed into cDNA either as full length clones or cDNA libraries within vectors. Full length cDNA clones of viruses that led to successful infectious viruses include tobacco mosaic virus (TMV) (Meshi *et al.,* 1986; Dawson *et al.,* 1986; Holt & Beachy, 1991), turnip yellow mosaic virus (TYMV) (Weiland & Dreher, 1989; Skotnicki *et al.,* 1992; Boyer *et al.,* 1994) and black queen cell virus (BQCV) (Benjeddou *et al.,* 2002). Variations on the cDNA technology have been used including improved vector primed strategies (Petty *et al.,* 1988) and synthetic DNA cassettes (Rizzo & Palukaitis, 1990). The development of reverse transcriptase's and DNA polymerases with increased fidelity and increases on the length that can be amplified has greatly benefited the development of infectious RNA clones (Boyer & Haenni, 1994). A very important aspect is the promoter sequences from which the infectious RNA is expected for *in vitro* or *in vivo* transcription because it directly affects the yield of transcripts (Boyer & Haenni, 1994). There

are several promoters which have been used from *E. coli*, for example Pm and promoters of bacteriophage SP6, T3 and T7. However obtaining full length cDNA clones or transcripts does not ensure biological activity in all cases. The infectivity of infectious clones is difficult to compare since results vary by multiple authors and also various methods used. For successful infection the viral transcripts have to mimic that of the virion RNA as closely as possible (Gritsun & Gould 1995).

More recently PCR has been the preferred choice in obtaining infectious clones of RNA viruses. These either include or exclude the use of vectors. Gritsun & Gould, (1995) used reverse transcribed cDNA from tick borne encephalitis virus (TBEV) and Benjeddou *et al.* (2002) for BQCV used PCR to amplify two halves of the genome namely the 5' half (non structural genes) and the 3' half (structural genes) using primers designed to these regions. The halves of the viruses were either ligated together or fused by fusion PCR to join the 5' half and the 3' half to obtain a full length clone of TBEV and BQCV respectively. With both of these methods point mutations can be included to introduce restriction enzymes sites for ligation or mutating the virus for identification purposes from the wild type. Infectious clone technology has been used to greatly advance the study of viruses. A step further is the development of chimeric viruses or genetically altered infectious viruses to help further the research of these viruses.

1.7.2 Chimeric viruses

Since infectious clone technology has become routine in molecular biology many other techniques have been used based on infectious clone technology. Also with rapid PCR technology and cloning, chimeric viruses can be obtained within days followed by the testing of the virus. Chimeric organisms have come about by researchers substituting genome regions across different viral genomes within the same family or different families of viruses (Dekker *et al.,* 2000). These substitutions give rise to chimeric recombinant viruses which have many different uses in molecular biology and other aspects of biology. Chimeric viruses are constructed by exchanging or substituting parts of viral genomes from one virus to another and observing if these newly formed viruses are viable (Pletnev & Men, 1998; Pletnev *et al.,* 1992; Kuhn *et al.,* 1996; Chambers *et al.,* 1999; Spielhofer *et al.,* 1998). Chimeric viruses have been used to better understand and also define specific functions of viral genomes, their contributions of different components to viral growth and virulence (Kuhn *et al.,* 1996; Powers *et al.,* 2000). Most importantly these viruses can be used to develop vaccines against the parental viruses (Pletnev *et al.,* 1992; Spielhofer *et al.,* 1998; Chambers *et al.,* 1999). Many studies have shown that virus activity being it virulence or replication can decrease by altering the virus. Kuhn *et al.* (1991) interchanged 3' and 5' non translated regions (NTRs) of a Sindbis (SIN) and Ross River virus (RRV). It resulted in the virus being attenuated. Furthermore the exchange of entire structural and non structural genes between SIN and RR virus resulted in

these chimeric viruses growing more poorly than parental viruses (Kuhn *et al.,* 1996).

Pletnev & Men, (1998) attempted to reduce the neurovirulence and abolish the neuro-invasiveness of the most virulent flavivirus, dengue virus (DEN) type 4. This was done by substituting structural and non structural regions from DEN type 4 to another flavivirus, tick borne, Langat virus (LGT). It was observed that the chimeras were 5000 times less neurovirulent than the parental LGT in mice. The results from this study were confirmed when the chimera appeared to be attenuated in monkeys and failed to infect permissive mosquito hosts. It was suggested that these chimeras were a promising vaccine candidate (Pletnev *et al.,* 2002).

Altering viruses at the genome level in order to study the impact of these viruses have been used with success. The ability to alter genomes of viruses has come about by years of research and now occurs routinely. The use of chimeric viruses to find vaccine candidates has great potential.

CHAPTER TWO

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

2.1 Abstract

A single multiplex reverse transcriptase (RT) PCR 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 primers were designed from the complete genome sequence to amplify fragments of 900bp from ABPV, 434bp from SBV and 316 bp from BQCV. Individual bee pupae crude homogenates or total RNA extracted from the crude extracts was 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. Field samples of apparently healthy pupae were screened for viral infections and evidence of inapparent virus infection and virus co-infections were found.

This chapter has been published as:
Topley, E.L., Davison, S., Leat, N. & Benjeddou, M. (2005). Detection of three honeybee viruses simultaneously by a single multiplex reverse transcriptase PCR. *African Journal of Biotechnology* **4**, 763-767

2.2 Introduction

The study of viral diseases of honeybees has become increasingly important to honeybee keepers and all related agricultural industries. Distributed worldwide these viruses together with pollution and the use of insecticides has resulted in high honeybee mortality rates which pose a real threat to these industries (Morse & Calderone, 2000; Spira, 2001). Currently there are 18 different honeybee viruses which have been identified and characterised with many of them persisting as inapparent infections, which cause sub-lethal infections in apparently healthy bees (Allen & Ball, 1996; Bailey *et al.*, 1983; Ball & Allen, 1988). Inapparent infections make viral diagnosis difficult due to the lack of gross symptoms visible to the eye (Bailey, 1965). The parasitic mite *Varroa destructor* (formerly named *Varroa jacobsoni*) has been suggested to play a pivotal role in honeybee mortality (Allen & Ball 1996; Bailey *et al.*, 1983; Ball & 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 & Allen, 1988; Bowen-Walker *et al.*, 1999) or activator (Ball & Allen, 1988; Brødsgaard *et al.*, 2000) of the inapparent viruses found in bees.

To date the genomes of six viruses 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), Kashmir bee virus (KBV) (de Miranda *et al.,* 2005), deformed wing virus (DWV) (GenBank accession no.: NC_004830). 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). All of these viruses are single stranded RNA viruses having 30 nm isometrical particles, and they are morphologically indistinguishable from each other (Allen & Ball, 1996). ABPV was first discovered as an inapparent infection during laboratory experiments (Bailey *et al*., 1963), and is spread by way of salivary gland secretions of adult bees, which are infected and in turn contaminate brood food stores (Ball, 1985). ABPV infections are known to be widespread if the colonies are infested with *Varroa destructor* (Allen & Ball, 1996; Ball & Allen, 1988). 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 & Woods, 1977) and is also very often detected when colonies are infested with the microsporidian mite *Nosema apis* (Allen & Ball, 1996; Bailey *et al.*, 1983). *N. apis* may be implicated in the mortality of honeybees when infected with the parasite (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 & 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 & Fernando, 1972). SBV has a single positive strand RNA genome consisting of 8832 nucleotides (Ghosh *et al.*, 1999).

Methods that are used to detect honeybee viruses include immunodiffusion techniques (Allen & 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 & Ball, 1996). However serological methods used have drawbacks when antiserum is raised to samples that contain virus mixtures. Evans (2001) found evidence that ABPV and Kashmir bee virus (KBV) can co-infect 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 & Shimanuki, 1999), SBV (Grabensteiner *et al*., 2000, BQCV and ABPV (Benjeddou *et al*., 2001, Evans & Hung, 2000). An advantage of RT PCR detection of honeybee viruses is the genetic comparison and classification of different virus strains that can 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.*, 2000; 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 & Jaykus, 1999), sugar beet viruses (Meunier *et al.*, 2003) and potato viruses (Nie & 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.

2.3 Materials and methods

2.3.1 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 *et al*. (2000).

2.3.2 RNA purification

Total RNA extraction was performed by adding 1 volume of 2% SDS (sodium dodecyl sulphate) (w/v) in 1 X 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).

2.3.3 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 14 000 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.

2.3.4 Oligonucleotide primers

The PCR primers were designed within a 1 kb region from the 3' end of each of the individual virus genomes (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 *et al*. (2001). Due to slight variations in the genome sequences of different strains of ABPV, SBV and BQCV deoxyinosine residues (denoted as I) were 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 percentage of sequence identity between primer and non corresponding genome sequences was 65% (data not shown). PCR conditions were optimised after extensive trails with primer ratios and annealing temperatures to enhance the amplification of each virus. Initially a primer pair was designed to amplify Kashmir bee virus (KBV) in the same genome region as the other viruses but due to primer interactions between ABPV and KBV RNA in the multiplex RT-PCR, KBV was subsequently removed from the RNA pools added to the multiplex reactions.

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

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

Table 2.1 Multiplex primers for detection of the three honeybee viruses

2.3.5 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 deoxynucleotide 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 was completed with a continuous RT-PCR method in a GeneAmp Perkin Elmer 2400 thermocycler. The cycle stages were: RT 58°C for 30min followed by denaturation at 94°C for 2 min. This was followed by 10 cycles of 94°C for 30s, 63°C for 30s and 68°C for 1 min. Then followed 25 cycles of 94°C for 30s, 63°C for 30s and 68°C for 1 min plus 5s 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.

2.3.6 Nucleotide sequencing and analysis

Single bands were excised from the gel and purified using the High Pure PCR product purification kit (Roche). Purified PCR products were then sequenced in both directions with the prime pairs 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.

2.3.7 Blind tests and natural infections

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

2.4 Results

Total RNA and extracted crude 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 900bp, SBV 434bp and BQCV 316bp. 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 2.2).

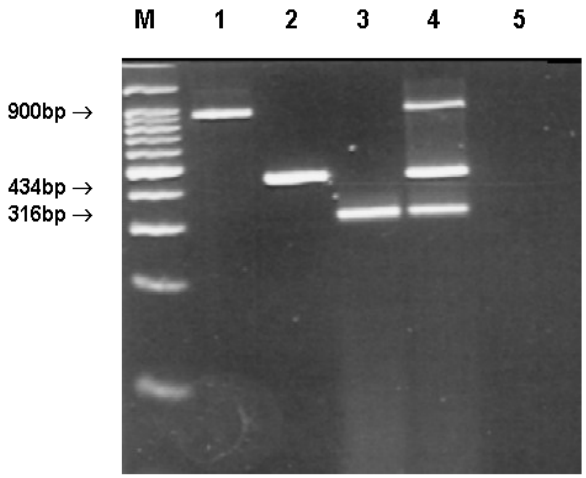


Figure 2.2
Reverse transcriptase (RT) PCR amplification of ABPV, SBV and BQCV in honeybees in a uniplex PCR and by Multiplex-RT-PCR. Extracted RNA from laboratory infected bee pupae were used with the corresponding primers for the particular virus for the uniplex PCR and a combination of primers for Multiplex-RT-PCR of the primer sets for ABPV, SBV and BQCV. Lane M, 100bp 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).

The results of the sequencing and BLAST searches of the PCR products showed that they belong to the corresponding ABPV, SBV or BQCV, with 97 to 98% identities to the particular reference strains contained in the GenBank database. 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 2.3 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.

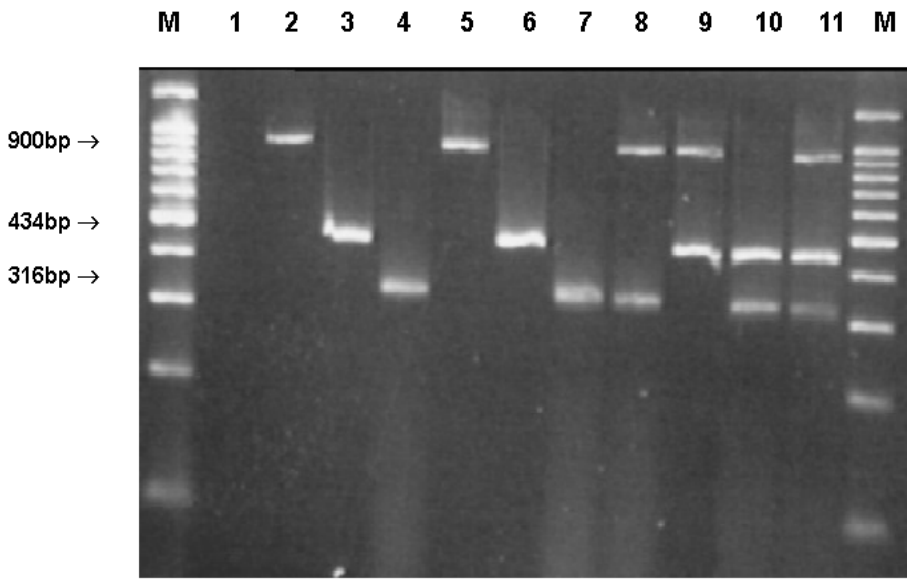


Figure 2.3
Reverse transcription (RT) PCR detection of ABPV, SBV, and BQCV in a uniplex PCR and as a Multiplex-RT-PCR to test the specificity of the primers sets for the three viruses. The specificity of the primers were tested on ABPV, SBV and BQCV virus stocks (positive controls) and laboratory infected pupae using a cocktail of primers in the M-RT-PCR. Lane M, 100bp 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).

A blind test was also carried out on forty samples of bees infected with ABPV, SBV, BQCV and apparently healthy bees. M-RT-PCR successfully identified the samples containing ABPV, SBV and BQCV in all instances.

This assay was further tested against field samples, to find out whether it would identify viruses present in bees under natural conditions. Forty field samples of randomly selected honeybee pupae, from the same colony were screened for viral infections, using this assay. Crude extracts of individual bee pupae were collected and used in the M-RT-PCR test. Of the samples thirty-four samples were negative, five samples tested positive for SBV and one sample tested positive for both BQCV and SBV.

2.5 Discussion

The assay tested in this chapter demonstrates the feasibility of M-RT-PCR using specific primers for the positive identification of several honeybee viruses in a single step reaction. The presented 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 & Shimanuki, 1999). It also has the potential to be used to study inapparent virus 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 could detect lower levels of virus in inapparently infected bees showing no signs of symptoms. However, field samples tested by this assay contained, what appeared to be apparently healthy honeybee pupae that were collected from a

colony that was previously infested with varroa to increase the chances of finding virus infections. The crude extracts of the individual bee pupae that were randomly selected from the same colony used in the M-RT-PCR, showed simultaneous and single infections as well as no infections. Of the forty samples tested thirty four samples presented negative results, in the remaining samples five tested positive for SBV and one sample was positive for both BQCV and SBV. It has been shown that the simultaneous detection of BQCV and SBV is not unusual and that the sample from which BQCV was first isolated was a mixed preparation that also contained SBV (Bailey & Woods 1977). It was suggested to be due to the presence of apparently healthy but SBV infected bee pupae used in the propagation of BQCV. However, Leat and co workers (Leat *et al*., 2000) argued that the simultaneous infection of these two viruses does exist and it is due to the viruses' wide distribution rather than there being a specific relationship between the two viruses. Dall (1985) also found no evidence of mixed infections in the case of KBV and SBV. Working on BQCV, KBV and SBV inapparent infections, Anderson & 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 that only one virus can be activated and multiply to detectable concentrations. The activated replicating virus would suppress the activation of any other viruses in the honeybee (Anderson & Gibbs 1988). In agreement with these arguments Evans (2001) presented evidence that ABPV and KBV could infect the same honeybee simultaneously and further noted that KBV levels appeared lower than ABPV.

The assay presented in this chapter can be further optimised and improved upon by adding more bee viruses as the genome sequences become available. It can prove to be a useful tool in studying inapparent viral infections as well as investigating suppression between viruses in the case of simultaneous infections. This M-RT-PCR assay can also be a rapid identification tool to be used to identify any viral infections in hives for the honeybee industry.

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 $(T)_{15}$ 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) from there the alignments were entered into a boxshade program at the Swiss institute of bioinformatics (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.

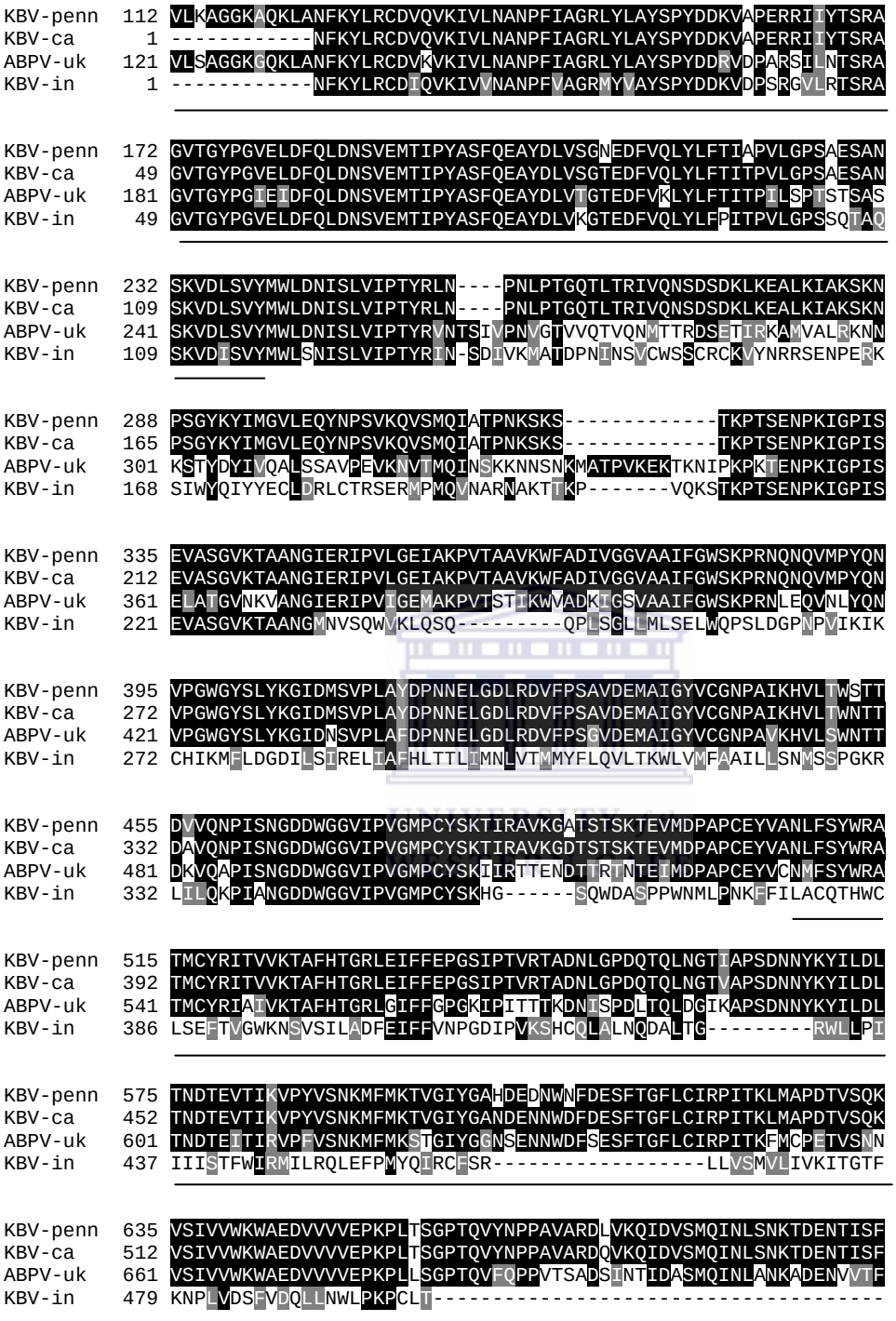


Figure 3.1

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.

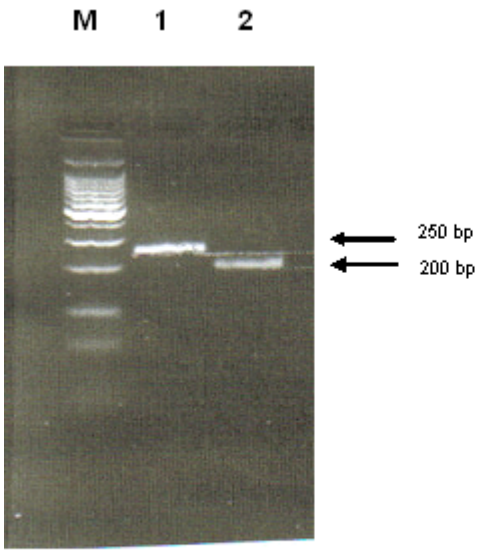


Figure 3.3

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 *Spodotera 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 inapparant 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 & Lomonossof, 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' TTTTTTTTTTTTTGCAAC 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 µl of RNase H (2 units/µl) 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 50µl with 2µl 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 (Pharmingen). 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).

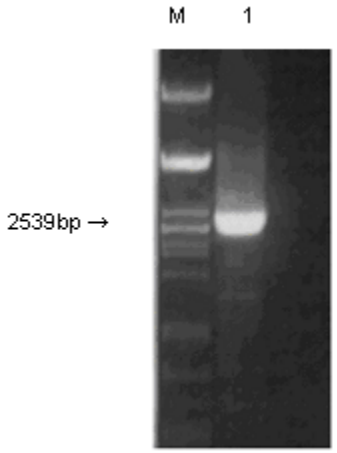


Figure 4.1
Amplification of the BQCV capsid gene with full length single stranded (ss) cDNA. Primers CAPF and CAPR introduced an ATG for initiation of replication in cell culture. M *Pst* lambda DNA marker; lane 1 BQCV capsid PCR product.

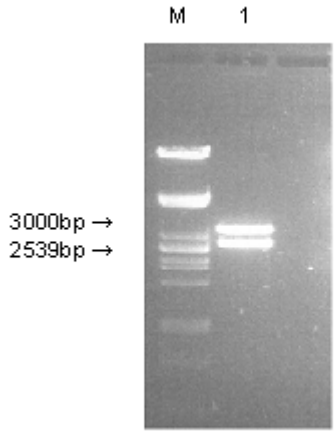


Figure 4.2
Plasmid DNA isolation and restriction digest of pGemT containing the BQCV capsid gene. The resulting pGemCAP plasmid was digested with *Bgl II*. Lane M, *Pst* lambda DNA marker; lane 1 pGem vector (3000bp) and BQCV capsid gene (2539bp);

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 recombined baculovirus containing the BQCV capsid protein was incubated at 27ºC 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).

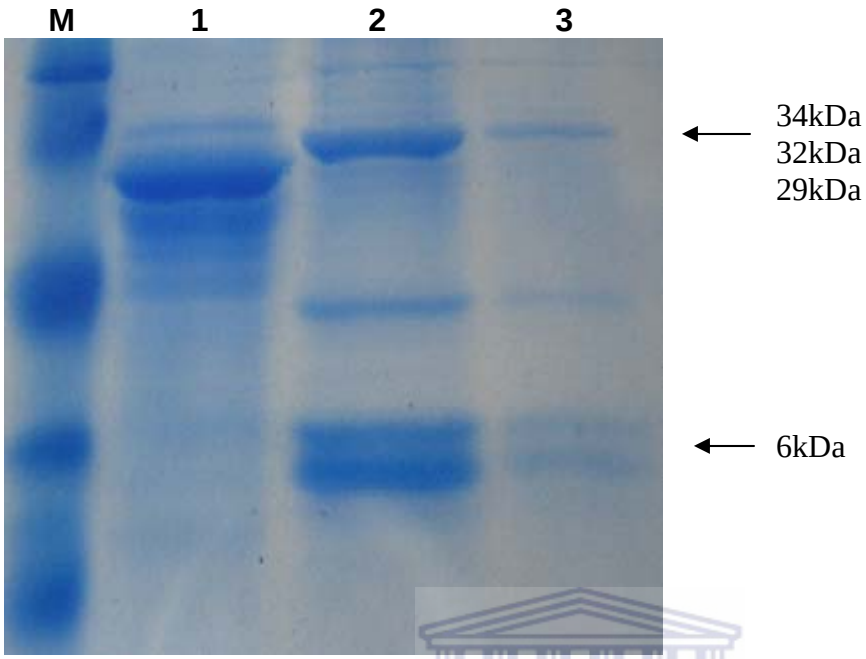


Figure 4.3

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.

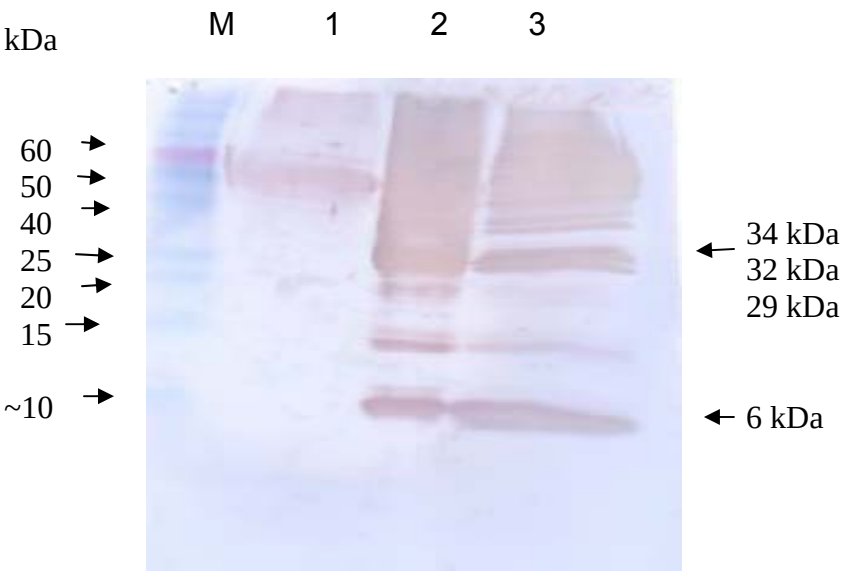


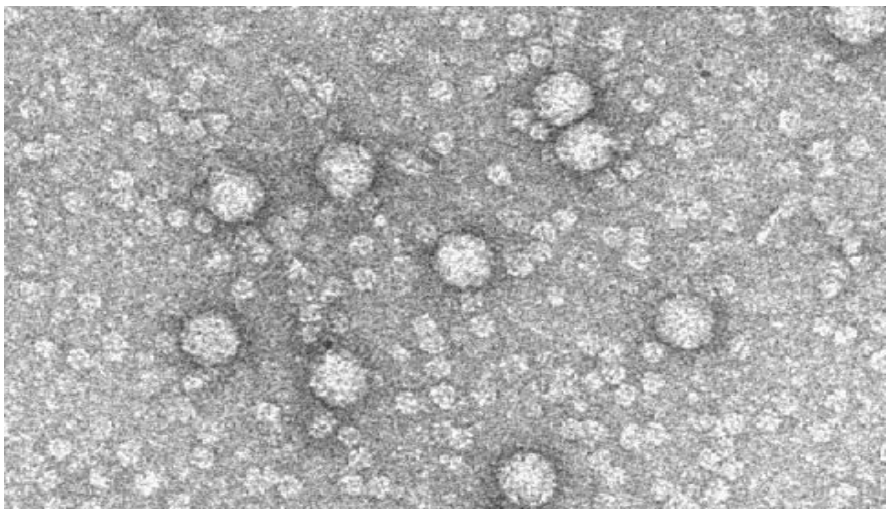
Figure 4.4

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 BCQV 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 nonstructural 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 *HiP*V 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 swopping 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' TTTTTTTAATTTACTAATTC 3	9462-9477
2*		5' TTTTTTTTTTTTTGCAAC 3'	8546-8563
3		5' GGGTAACCATGTTGTGTTGCGATTCCCAAA CTACTCATAACCTGAAAGGCCAAGAGCAATC 3'	6322-6355 5595-5628
4		5' GATTGCTCTTGGCCTTTCAGGTTATGAGTAGTT TGGGAATCGCAACACAACATGGTTACCC 3'	5595-5628 6322-6355
5*	*KpnI*	5' GGGTAT**GGTACC**$(T)_{25}$GCAACAAGAAGAAACGT AAACC 3'	8529-8575
6	*NotI*	5' GGGTATGCGGCCGC $(T)_{40}$ AATTTACTAATTCG AAATTTTGACGC 3'	9445-9531
7	*NotI*	5' GGGTAT**GCGGCCGC***ATTTAGGTGACACTATAGA ATA*CCCGTCAAAATAACAACTTATAACAC 3'	1-26
8		5' GCTCAGGAGAGATTCCTAAATTACTACTTGTAA TTTCTTGACTTCTCTTAAAACCAACAATG 3'	6239-6328 5627-5655
9		5' AAGATCACATTGTTGGTTTTAAGAGAAGTCAAGA AATTACAAGTAGTAATTTAGGAATCTC 3'	5627-5662 6301-6328
10*	*NotI*	5' GGGTAT**GCGGCCGC***ATTTAGGTGACACTATAGA ATA*CGCAAAATTGCGTATAGTATATAAAT 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^{PLUS} 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 µl. Each reaction contained 2 µl template, 0.5 mM of each deoxynucleotide triphosphate, 0.3 µM forward primer 10 and 0.3 µM reverse primer 3, 5 µl 10 X PCR buffer and 0.75 mM $MgCl_2$. 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

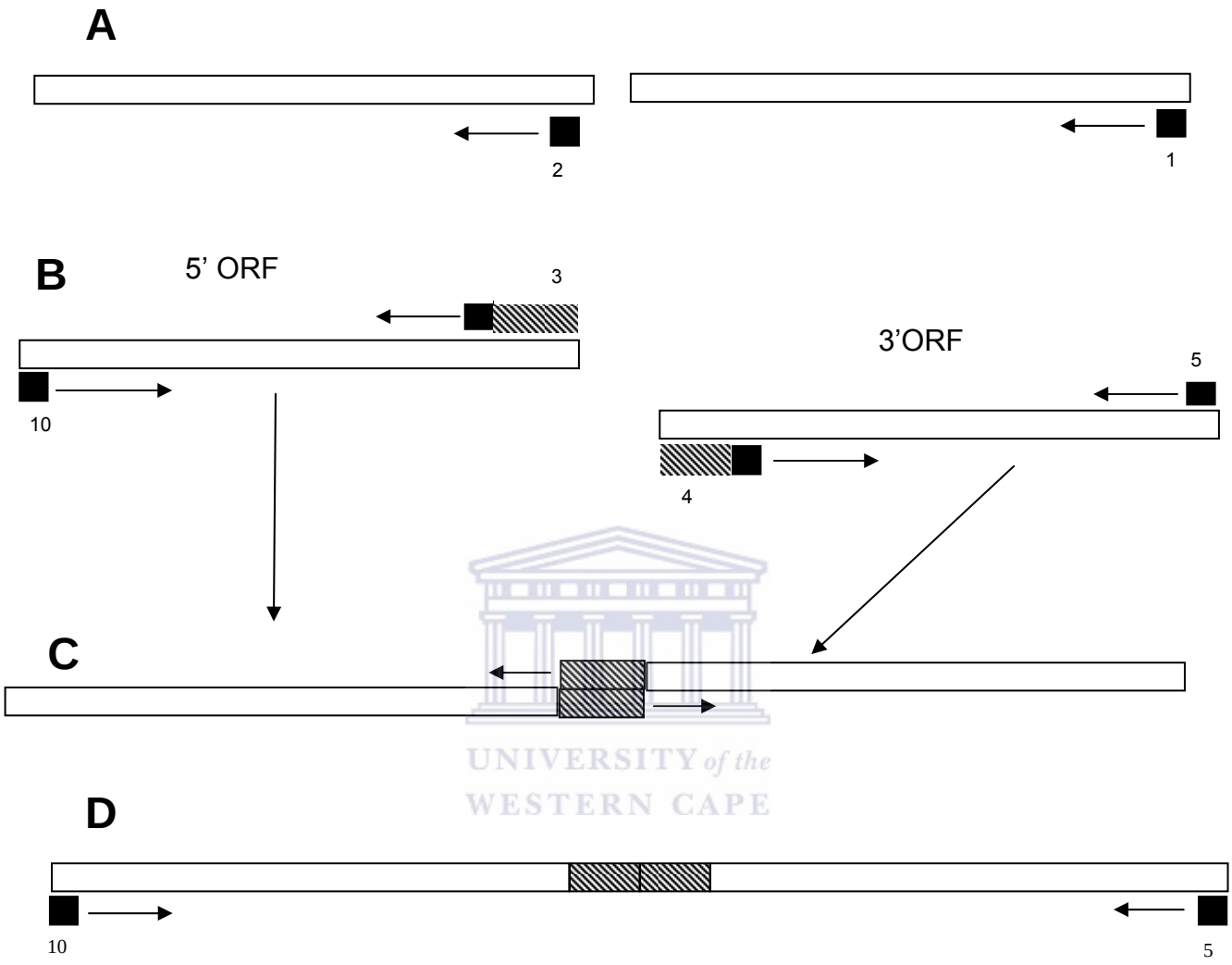


Figure 5.1

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.

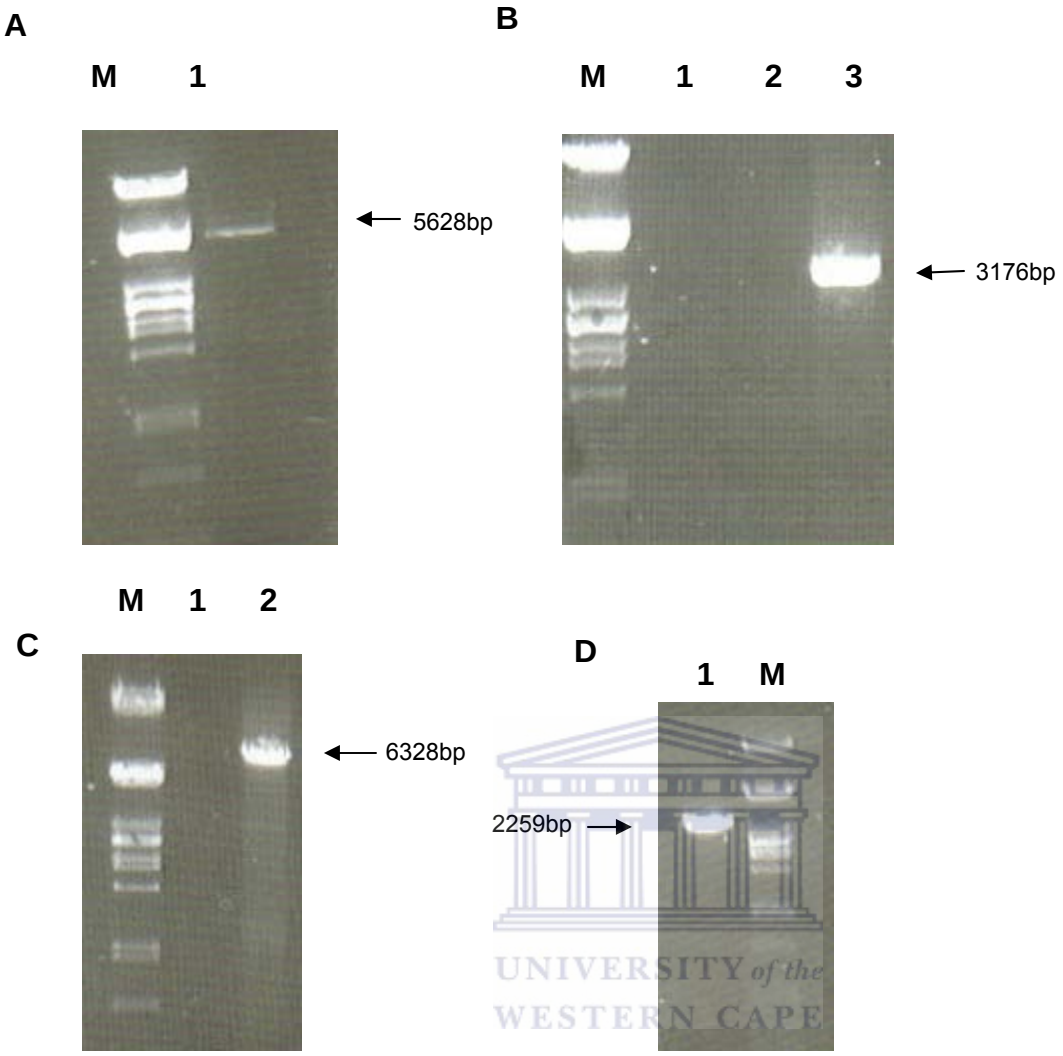


Figure 5.2

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 vesticular 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.

REFERENCE

Aguilar, J.C., Pérez-Breña, M.P., Garcia, M.L., Cruz, N., Erdman, D.D. & Echevarría, J.E. (2000). Detection and identification of human parainfluenza viruses 1, 2, 3 and 4 in clinical samples of pediatric patients by multiplex reverse transcription PCR. *J. Clin. Microbiol.* **38**, 1191-1195.

Allen, M.F. & Ball, B.V. (1995). Characterisation and serological relationship of strains of Kashmir bee virus. *Ann. Appl. Biol.* **126,** 471-484.

Allen, M. & Ball, B. (1996). The incidence and world distribution of honeybee viruses. *Bee World* **77**, 141-162.

Allen, M.F., Ball, B.V., White, R.F. & Antoniw, J.F. (1986). The detection of acute paralysis virus in *Varroa jacobsoni* by the use of a simple indirect ELISA. *J. Apicul. Res.* **25**, 100-105

Allsopp, M. (1997). The honeybee parasitic mite *Varroa jacobsoni* in South Africa. *South African Bee Journal* **69** (4), 73-82.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Anderson, D.L. (1984). A comparison of serological techniques for detecting and identifying honeybee viruses. *J. Invertebr. Pathol*. **44**, 233-243.

Anderson, D.L. (1991). Kashmir bee virus – a relatively harmless virus of honeybee colonies. *Am. Bee J*. **131**, 767-770.

Anderson, D.L. (1993). Pathogens and queen bees. *Australasian Beekeeper* **94**, 292-296.

Anderson, D.L. (2000). Variation in the parasitic bee mite *Varroa jacobsoni* Oud. *Apidologie*. **31**, 281-292.

Anderson, D.L. & Gibbs, A.J. (1988). Inapparant virus infections and their interactions in pupae of the honey bee (*Apis Mellifera* Liinnaeus) in Australia. *J. Gen. Virol.* **69**, 1617-1625.

Anderson, D.L. & Trueman, J.W.H. (2000). *Varroa jacobsoni* (Acari:Varroidae) is more than one species. *Exp. Appl. Acarol.* **24,** 165-189.

Bailey, L. (1965). The occurrence of chronic acute paralysis viruses in bees outside of Britain. *J. Invertebr. Pathol.* **7,** 167-169.

Bailey, L. (1969). The multiplication and spread of sacbrood virus of bees. *Ann. Appl. Biol.* **63**, 483-491.

Bailey, L. (1983). *Melissococcus pluton*, the cause of European foul brood of honeybees (*Apis* spp.). *J. Appl. Bacteriol*. **55,** 65-69.

Bailey, L. & Fernando, E.F.W. (1972). Effects of sacbrood virus on adult honeybees. *Ann. Appl. Biol.* **72**, 27-35.

Bailey, L. & Gibbs, A.J. (1964). Acute infection of bees with paralysis virus. *J. insect Pathol*. **6**, 395-407.

Bailey, L. & Woods, R.D. (1974). Three previously undescribed viruses from the honey bee. *J. Gen. Virol.* **25**, 174-186

Bailey, L. & Woods, R.D. (1977). Two more small RNA viruses from honeybees and further observations on sacbrood and acute paralysis viruses. *J.Gen. Virol*. **25**, 175-186.

Bailey, L., Ball, B. & Woods, R.D. (1963). Two viruses from adult honeybees (*Apis mellifera* Linnaeus). *Virology* **23,** 425-429.

Bailey, L., Ball, B.V. & Perry, J.N. (1983). Association of viruses with two protozoal pathogens of the honey bee. *Ann. Appl. Biol.* **103**, 13-20.

Bailey, L., Carpenter, J.M. & Woods, R.D. (1979). Egypt bee virus and Australian isolates of Kashmir bee virus. *J. Gen. Virol.* **43**, 641-647.

Bailey, L., Gibbs, A.J. and Woods, R.D. (1964). Sacbrood virus of the larval honeybee (*Apis mellifera* Linnaeus). *Virology* **23**, 425-429.

Bakonyi, T., Farkas, R., Szendroi, A., Dobos-Kovacs, M. & Rusvai, M. (2002). Detection of Acute bee paralysis virus by RT-PCR in honeybee and *Varroa destructor* field samples: rapid screening of representative Hungarian apiaries. *Apidologie* **33**, 63-74.

Ball, B.V. (1985). Acute paralysis virus isolates from honeybee colonies infested with Varroa jacobsoni. *J. Apicult. Res.* **24**, 115-119.

Ball, B.V. (1989). *Varroa jacobsoni* as a virus vector. In Present *Status of Varroatosis in Europe and progress in the Varroa mite control*, pp 241-244. Edited by R. Cavalloro. Brussells: ECSC-EEC-EAEC.

Ball, B.V. (1993). The damaging effects of *Varroa jacobsoni* infestation. In *Living with varroa*, pp.9-17. Edited by A. Matheson. Proceedings of the IBRA

symposium, London 21 November 1992, International Bee Research Association, Cardiff.

Ball, B. (1997). Varroa and viruses. In Varroa! Fight the mite, pp. 11-15. Edited by P. Munn and R. Jones. Cardiff: International Bee Research Association.

Ball, B.V. & Allen, M.F. (1988). The prevalence of pathogens in honeybee (*Apis mellifera*) colonies infested with parasitic mite *Varroa jacobsoni*. *Ann. Appl. Biol*. **113**, 237-244.

Ball, B.V. & Bailey, L. (1997). Viruses. In *Honeybees pests, predators and diseases*, pp11-32. Edited by R.A. Morse and K. Flottum. A.I. Root Company, Medina.

Beljelarskaya, S.N. (2002). A baculovirus expression system for insect cells. *Mol. Biol.* **36** (3), 281-292.

Belyaev, A.S. & Roy, P. (1993). Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus-like particles in insect cells. *Nucleic acids Res.* **21**, 1219-1223

Benjeddou, M., Leat, N., Allsopp, M. & Davison, S. (2001). Detection of acute paralysis virus and black queen cell virus from honeybees by reverse transcriptase PCR. *Appl. Environ. Microb.* **67**, 2384-2387

Benjeddou, M., Leat, N., Allsopp, M. & Davison, S. (2002). Development of infectious transcripts and genome manipulation of Black queen cell virus of honeybees. *J. Gen. Virol.* **83**, 3139-3146

Bertolini, E., Olmos, A., Martinez, C., Gorris, M.T. & Cambra, M. (2001). Single-step multiplex RT-PCR for simultaneous and colourmetric detection of six RNA viruses in olive trees. *J. Virol. Methods* **96**, 33-41.

Bowen-Walker, P.L., Martin, S.J. & Gunn, A. (1999). The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasite mite *Varroa jacobsoni* Oud. *J. Invertebr. Pathol* **73**, 101-106

Boyer, J-C. & Haenni, A-L. (1994). Infectious transcripts and cDNA clones of RNA viruses. *Virology* **198**, 415-426.

Boyer, J.C., Drugeon, G., Seron, K., Morch-Devignes, M.D., Agnes, F. & Haenni, A.L. (1993). In vitro transcripts of turnip yellow mosaic virus encompassing a long 3' extension or produced from a full-length cDNA clone harbouring a 2-kb long PCR amplified segment are infectious. *Res. Virol.* **144**, 339-348.

Brødsgaard, C.J., Ritter, W., Hansen, H. & Brodsgaard, H.F. (2000). Interactions among *Varroa jacobsoni* mites, acute paralysis virus and *Paenibacillus larvae larvae* and their influence on mortality of larval honeybees *in vitro*. *Apidologie* **31**, 543-554

Buys, B. (1995). First record in South Africa of the tracheal mite *Acarapis woodii*. *South African Bee Journal* **67** (3), 75-78.

Chambers, T.J., Nestorowicz, A., Mason, P.W. & Rice, C.M. (1999). Yellow Fever/Japanese Encephalitis Chimeric Viruses: Construction and Biological Properties. *J. Virol.* **73** (4), 3095-3101.

Chantawannakul, P., Ward, L., Boonham, N. & Brown, M. (2006). A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in

Varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary. *J. Invertebr. Pathol.* **91**, 69-73.

Chen, Y. P., Higgins, J.A. & Feldlaufer, M.F. (2005). Quantitative real-time transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). *Appl. Environ. Microb.* **71**, 436-441.

Chen, Y., Zhao, Y., Hammond, J., Hsu, H., Evans, J. & Feldlaufer, M. (2004). Multiple virus infection in the honey bee and genome divergence of honey bee viruses. *J. Invertebr. Pathol.* **87**, 84-93.

Christian, P., Carstens, E., Domier, L., Johnson, K., Nakashima, N., Scotti, P. & van der Wilk, F. (2002). Infectious flacherie-like viruses. ICTV Virus Taxonomy 2002. Available at www.ictvdb.iacr.ac.uk/Ictv/index.htm.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, Evans, J.D., Moran, N.A., Quan, P., Briese, T., Hornig, M., Geiser, D.M. & other authors. (2007). A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* 318 pp. 283-286.

Crawford, S.E., Estes, M.K., Barone, C., O'Neal, C.M., Cohen, J. & Conner, M.E. (1999). Heterotypic protection and induction of a broad heterotypic neutralization response by rotavirus-like particles. *J. Virol.* **73**, 4813-4822.

Dall, J.D. (1985). Inapparant infection of honey bee pupae by Kashmir & sacbrood viruses in Australia. *Ann. Appl. Biol.* **106**, 461-468.

Davies, A.H. (1994). Current methods for manipulating baculoviruses. *Nature Biotech*. **12**, 47-50.

Davies, A.H. (1995). Advances in the use of recombinant baculoviruses for the expression of heterologous proteins. *Curr. Opin. Biotech.* **6**, 543-547.

Dawson, W.O., Beck, D.L., Knorr, D.A. & Grantham, G.L. (1986). cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts*. PNAS USA* **83,** 1832-1836.

De Jong, D. & De Jong, P.H. (1983) Longevity of Africanized Honey Bees (*Hymenoptera: Apidae*) infested by *Varroa jacobsoni* (*Parasitiformes:Varroidae*). *J. Econ. Entomol.* **7** (4), 766-768.

Dekker, A., Leendertse, C.H., van Poelwijk, F., Rebel, J.M.J. & Moormann, R.J.M. (2000). Chimeric swine vesticular disease virus produced by fusion PCR: a new method for epitope mapping. *J. Virol. Methods* **86**, 131-141.

Delfinado, M.D. (1963). Mites of the honeybee in Southeast Asia. *J. Apicult. Res*. **2**, 113-114.

de Miranda, J.R., Drebot, M., Tyler, S., Shen, M., Cameron, E., Stoltz, D.B. & Camazine, S.M. (2004). Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. *J. Gen. Virol.* **85**, 2263-2270.

Derakhshifar, I., Moosbeckhofer, R., Licek, E. & Nowotny, N. (2000). Sacbrood virus of the honeybee (*Apis mellifera*): Rapid identification and phylogenetic analysis using reverse transcriptase PCR. *Clinical Diagnostic Laboratory Immunology* **8**, 93-104.

Dietz, A. (1992). Honeybees of the world. In *The hive and the honeybee*, 10th edn, Edited by J.M. Graham. Hamilton, Illinois: Dadant & Sons.

Domier, L.L, Mc Coppin, N.K. & D'Arcy, C.J. (2000). Sequence requirements for the translation initiation of *Rhopalosiphum padi* virus ORF2. *Virology* **268**(2), 264-271.

Echevarría, J.E., Erdman, D.D., Swierkosz, E.M., Holloway, B.P. & Anderson, L.J. (1998). Simultaneous detection and identification of human parainfluenza viruses 1, 2 and 3 from clinical samples by multiplex PCR. *J. Clin. Microbiol.* **36**, 1388-1391.

Evans, J.D. (2001). Genetic evidence for co-infection of honeybees by acute bee paralysis virus and Kashmir bee viruses. *J. Invertebr. Pathol.* **78**, 189-193.

Evans, J.D. & Hung, A.C. (2000). Molecular phylogenetics and the classification of honey bee viruses. *Arch. Virol.* **145**, 2015-2026.

Finely, J., Camazine, S. & Frazier, M. (1996). The epidemic of honeybee colony losses during the 1995-1996 season. *Am. Bee J.* **136**, 805-808.

Ghosh, R.C., Ball, B.V., Wilcocks, M.M. & Cater, M.J. (1999). The nucleotide sequence of sacbrood virus of the honeybee: an insect picorna-like virus. *J. Gen. Virol.* **80**, 1541-1549.

Govan, V.A., Leat, N., Allsopp, M. & Davison, S. (2000). Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect infecting RNA viruses. *Virology* **277**, 457-463.

Grabensteiner, E., Ritter, W., Carter, M. J., Davison, S., Pechhacker, H.,. Kolodziejek, J., Roeking, 0., Derakhshifar, I., Moosbeckhofer, R., Licek, E. & Nowotny, N. (2000). Sacbrood virus of the honeybee (*Apis mellifera*): rapid identification and phylogenetic analysis using reverse transcription-PCR. *Clin. and Diag. Lab. Immun.* **8** (1), 93-104.

Gritsun, T.S. & Gould, E.A. (1995). Infectious tick borne encephalitis virus generated in days by RT PCR. *Virology* **214**, 611-618

Hegedus, D.D., Pfeifer, T.A., Hendry, J., Theilmann, D.A. & Grigliatti, T.A. (1997). A series of broad range shuttle vectors for constitutive and inducible expression of heterologous proteins in insect cell lines. *Gene* **207**, 241-249.

Holt, C.A. & Beachy, R.N. (1991). *In vivo* complementation of infectious transcripts from mutant tobacco mosaic virus cDNAs in transgenic plants. *Virology* **181**, 109-117.

Hung A.F.C. & Shimanuki, H. (1999). A scientific note on the detection of Kashmir bee virus in individual honeybees and *Varroa jacobsoni* mites. *Apidologie* **30**, 353-354.

Hung, A. C. F., Shimanuki, H. & Knox, D. A. (1996). Inapparent infection of acute paralysis virus and Kashmir bee virus in the U.S. honey bees. *Am. Bee J.* **136**, 874-876.

Huynh, C.Q. & Zieler, H. (1999). Construction of modular and versatile plasmid vectors for the high-level expression of single or multiple genes in insects and insect cell lines. *J. Mol. Biol.* **288**, 13-20

Igarashi, T., Ami, Y., Yamamoto, H., Shibata, R., Kuwata, T., Mukai, R., Shinohara, K., Komatsu, T., Adachi, A. and Hayami,M. (1997). Protection of monkeys vaccinated with vpr- and/or nef-defective simian immunodeficiency virus strain mac/human immunodeficiency virus type 1 chimeric viruses: a potential candidate live-attenuated human AIDS vaccine. *J. Gen. Virol.* **78**, 985 – 989.

Jarvis, D.L., Weinkauf, C. & Guarino, L.A. (1996). Immediate-early baculovirus vectors for foreign gene expression in transformed or infected insect cells. *Protein Expres. Purif.* **8**, 191-203.

Jiang, X., Wang, M., Graham, D.Y. & Estes, M.K. (1992). Expression, Self assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* **66**(11), 6527-6532.

Johannesmeier, M.F. & Mostert, A.J.N. (2001). Crop pollination. In *Beekeeping in South Africa*, 3rd edn, pp. 235-250. Plant Protection Handbook No. 14. Edited by M. F. Johannesmeier Pretoria: Agricultural Research Council.

Ke, A., Gillock, E.T., Sweat, J.A. Reeves, W.M. & Consigli, R.A. (1999). Use of the baculovirus system to assemble polyomavirus capsid-like particles with different polyomavirus structural proteins: analysis of the recombinant assembled capsid-like particles. *J. Gen. Virol.* **80**, 1009-1016.

Kitts, P.A., Ayres, M.D. & Possee, R.D. (1990). Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res.* **18**, 5667-5672.

Kitts, P.A. & Possee, M.D. (1993). A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* **14**, 810-817.

Korth, K.L. & Levings, C.S. (1993). Baculovirus expression of the maize mitochondrial protein URF13 confers insecticidal activity in cell cultures and larvae. *PNAS USA* **90**, 3388-3392.

Kost, T.A. & Condreay, J.P. (1999). Recombinant baculoviruses as expression vectors for insect and mammalian cells. *Curr. Opin. Biotech.* **10**, 428-433.

Kraus, B. & Page, R.E. (1995). Effect of *Varroa jacobsoni* (*Mesostigmata:Varroidae*) on feral *Apis mellifera* (*Hymenoptera:Apidae*) in California. *Environ. Entomol.* **24** (6), 1473-1480.

Kuhn, R.J., Niesters, H.G., Hong, Z. & Strauss, J.H. (1991). Infectious RNA transcripts from Ross River virus cDNA clones and the construction and characterisation of defined chimeras with Sindbis virus. *Virology* **182**, 430-441.

Kuhn, R.J., Griffin, D.E., Owen, K.E., Niesters, H.G.M. & Strauss, J.H. (1996). Chimeric Sindbis- Ross River viruses to study interactions between alphavirus nonstructural and structural genes. *J. Virol.* **70**, 7900-7909.

Lanzi, G., de Miranda, J.R., Boniotti, M.B., Cameron, C.E., Lavazza, A., Capucci, L., Camazine, S.M. & Rossi, C. (2006). Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera* L.). *J. Virol.* **80**, 4998-5009.

Leat, N., Ball, B.V., Govan, V. & Davison, S. (2000). Analysis of the complete genome sequence of black queen cell virus, a picorna-like virus of honey bees. *J. Gen. Virol.* **81**, 2111-2119.

Lerch, R.A, & Friesen, P.D. (1993). The 35-kilodalton protein gene (p35) of *Autographa californica* nuclear polyhedrosis virus and the neomycin resistance gene provide dominant selection of recombinant baculoviruses. *Nucleic Acids Res.* **21**, 1753–1760.

Maori, E., Lavi, S., Mozes-Koch, R., Gantman, Y., Peretz, Y., Edelbaum, O., Tanne, E., & Sela, I. (2007). Isolation and characterisation of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to the intra- and inter- species recombination*. J.Gen. Virol.* **88**, 3428-3438.

Maree, S., Durbach, S. & Huismans, H. (1998). Intracellular production of African Horsesickenss virus core-like particles by expression of the two major core proteins, VP3 and VP7, in insect cells. *J. Gen. Virol.* **79**, 333-337.

Martin, S. J. (2001). The role of Varroa and viral pathogens in the collapse of honeybee colonies: a modelling approach. *J. Appl. Ecol.* **38**, 1082-1093.

Mayo, M.A. (2002). Virology division news: Virus taxonomy-Houston 2002. *Arch. Virol.* **147**, 1071-1076.

McCormick, C.J., Rowlands, D.J. & Harris. M. (2002). Efficient delivery and regulable expression of hepatitis C virus full-length and minigenome constructs in hepatocyte-derived cell lines using baculovirus vectors. *J. Gen. Virol.* **83**, 383-394

Medin, J.A., Hunt, L., Gathy, K., Evans, R.K. & Coleman, S. (1990). Efficient, low cost protein factories: Expression of human adenosine deaminase in baculovirus-infected insect larvae. *PNAS USA* **87**, 2760-2764.

Meshi, T., Ishikawa, M., Motoyoshi, F., Semba, K. & Okada, Y. (1986). *In vitro* transcription of infectious RNAs from full length cDNAs of tobacco mosaic virus. *PNAS USA* **81**, 1966-1970.

Meulenberg, J. J. M., Bos-de Ruijter, J. N. A., van de Graaf, R., Wensvoort, G. & Moormann, R. J. M. (1998). Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. *J. Virol.* **72**, 380-387.

Meunier, A., Schmit, J., Stas, A., Kutluk, N. & Bragard, C. (2003). Multiplex reverse transcription-PCR for the simultaneous detection of Beet necrotic yellow

vein virus, Beet *soilborne* virus and *Beet virus Q* and their vector *Polymyxa betae* KESKIN on sugar beet. *Appl. Environ. Microb.* **69**, 2356-2360.

Morse, R.A. & Calderone, N.W. (2000). The value of the honeybee pollination in the United States. *Bee Culture* **128**, 1-15.

Mortola, E. & Roy, P. (2004). Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. *FEBS letters* **576**, 174-178

Newcomb, W.W., Homa, F.L., Thomsen, D.R. Booy, F.P., Trus, B.L., Steven, A.C., Spencer, J.V. & Brown, J.C. (1996). Assembly of the herpes simplex virus capsid: characterisation of intermediates observed during cell-free capsid assembly. *J. Mol. Biol.* **263**, 432-446.

Nie, X. & Singh, R.P. (2000). Detection of multiple potato viruses using an oligo (dT) as a common cDNA primer in multiplex RT-PCR. *J. Virol. Methods* **86**, 179-185.

Nixon, M. (1982). Preliminary world maps of honey bee diseases and parasites. *Bee World* **63**, 23-42.

Norton, R.A., Caserta, M.T., Breese Hall, C., Schnabel, K., Hocknell, P. & Dewhurst, S. (1999). Detection of human herpesvirus 6 by reverse transcription-PCR. *J. Clin. Microbiol.* **37**, 3672-3675.

Olivier, V., Blanchard, P., Chaouch, S., Lallemand, P., Schurr, F., Celle, O., Dubios, E., Tordo, N., Thiéry, R., Houglgatte, R. & Ribière, M. (2008). Molecular characterisation and phylogenetic analysis of chronic bee paralysis virus, a honeybee virus. *Virus Res.* **132**, 59-68.

Ongus J.R., Peters D., Bonmatin J.M., Bengsch E., Vlak J.M. & van Oers M.M. (2004). Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. *J. Gen. Virol*. **85**, 3747-3755.

Oudemans, A.C. (1904). On a new genus and species of parasitic acari. Notes Leyden Museum. **24**, 216-222.

Petty, I.T.D., Hunter, B.G. & Jackson, A.O. (1988). A novel strategy for one-step cloning of full-length cDNA and its application to the genome of barley stripe mosaic virus. *Gene* **74**, 423-432.

Pletnev, A.G. & Men, R. (1998). Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4. *PNAS USA* **95**, 1746–1751.

Pletnev, A.G., Bray, M., Huggins, J. & Lai, C.J. (1992). Construction and characterization of chimeric tick-borne encephalitis/dengue type 4 viruses. *PNAS USA* **89**, 10532-10536.

Powers, A.M., Brault, A.C., Kinney, R.M. & Weaver, S.C. (2000). The use of chimeric Venezuelan Equine Encephalitis viruses as an approach for the molecular identification of natural virulence determinants. *J. Virol.* **74**, 4258-4263.

Pringle, F.M., Kalmakoff, J. & Ward, V.K. (2001). Analysis of the capsid processing strategy of *Thosea asigna* virus using baculovirus expression of virus-like particles. *J. Gen. Virol.* **82**, 259-266.

Pullen, S.S. & Freisen, P.D. (1994). Early transcription of the *ie*-1 transregulator gene of the *Autographa californica* nuclear polyhedrosis virus is regulated by DNA sequences within its noncoding leader region. *J. Virol.* **69**, 156-165.

Rebel, J.M.J., Leenderste, C.H., Dekker, A., van Poelwijk, F. & Moolman, R.J.M. (2000). Construction of full-length infectious cDNA clone of swine vesicular disease virus strain NET/ I/ 92 and analysis of new antigenic variants derived from it. *J. Gen. Virol.* **81**, 2763-2769.

Ritter, W. & Ruttner, F. (1980). Neue Wege in der behandlung der Varroatose. *AllgDtsch Imlerztg* **14**, 151-155.

Rizzo, T.M. & Palukaitis, P. (1990). Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2, and 3: Generation of infectious transcripts. *Molecular General Genetics* **222**, 249-256.

Rohrmann, G.F. (2008). Baculovirus Molecular biology [Internet]. Bethseda (MD). National library of Medicine (US). NCBI. Available at www. ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=bacvir

Roosien, J., Belsham, G.J., Ryan, M.D., King, A.M.Q. & Vlak, J.M. (1990). Synthesis of foot-and-mouth disease virus capsid proteins in insect cells using baculovirus expression vectors. *J. Gen. Virol.* **71**, 1703-1711.

Rosenfield, S.I. & Jaykus, L. (1999). A multiplex reverse transcription polymerase chain reaction method for the detection of foodborne viruses. *J. Food Protect.* **62**, 1210-1214.

Sambrook, J., Fritsh, E.F. & Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press. *Cold Spring Harbor*, N.Y.

Sasaki, J. & Nakashima, N. (1999). Translation initiation at the CUU codon is mediated by the internal ribosome entry site of an insect picorna-like virus *in vitro. J. Virol.* **73**, 1219-1226

Scott-Dupree, C & McCarthy, J. (1995). Honeybee viruses. *Bee Culture* **123**, (7) 392-396.

Seifer, M., Hamatake, R., Bifano, M & Standring, D.N. (1998). Generation of replication-competent hepatitis B virus nucleocapsids in insect cells. *J. Virol.* **72**, 2765-2776.

Shanks, M. & Lomonossoff, G.P. (2000). Co-expression of the capsid proteins of *Cowpea* mosaic virus in insect cells leads to the formation of virus like particles. *J. Gen. Virol.* **81**, 3093-3097.

Shen, M., Cui, L., Ostiguy, N. & Cox-Foster, D. (2005). Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and parasitic varroa mite. *J. Gen. Virol*. **86**, 2281-2289.

Shevchuk, N.A., Bryksin, A.V., Nusinovich, Y.A., Cabello, F.C., Sutherland, M. & Ladisch, S. (2004). Construction of long DNA molecules using long PCR based fusion of several fragments simultaneously. *Nucleic Acids Res.* **32**, e19, doi 10.1093/nar/gnh014.

Shimanuki, H., Calderone, N.W. & Knox, D.A. (1994). Parasitic mite syndrome: the symptoms. *Am. Bee J.* **134**, 827-828

Singh, R. (1998). Reverse transcriptase polymerase chain reaction for the detection of viruses from plants and aphids. *J. Virol. Methods* **74**, 125-138.

Singh, R., Nie, X. & Singh, M. (2000). Duplex RT PCR: reagent concentrations at reverse transcription stage affect the PCR performance. *J. Virol. Methods* **86**, 121-129.

Skotnicki, M., MacKenzie, A.M. & Gibbs, A.J. (1992). Turnip yellow mosaic virus variants produced from DNA clones encoding their genomes. *Arch. Virol.* **127**, 25-35.

Spielhofer, P., Bāchi, T., Fehr, T., Christiansen, G., Cattaneo, R., Kaelin, K., Billeter, M.A. & Naim, H.Y. (1998). Chimeric Measles Viruses with a Foreign Envelope. *J. Virol.* **72**, 2150-2159.

Spira, T.P. (2001). Plant pollinator interactions: a threatened mutualism with implications for the ecology and management of rare plants. *Nat. Areas J.* **21**, 78-88.

Steininger, C., Aberle, S.W. & Popow-Kraupp, T. (2000). Early detection of acute rhinovirus infections by rapid reverse transcription PCR assay. *J. Clin. Microbiol.* **39**, 129-133.

Stoltz, D., Shen, S.R., Boggis, C. & Sisson, G. (1995). Molecular diagnosis of Kashmir bee virus infection. *J. Apicult. Res.* **34**,153-160

Swart, D. J., Johannsmeier, M. F., Tribe, G. D. & Kryger, P. (2001). Diseases and pests of honeybees. In *Beekeeping in South Africa*, 3rd edn, pp. 198-222. Plant Protection Handbook No. 14. Edited by M. F. Johannsmeier Pretoria: Agricultural Research Council.

Tate, J., Liljas, L., Scotti, P., Christain, P., Lin, T. & Johnson, JE. 1999. The crystal structure of cricket paralysis virus: the first view of a new virus family. *Nat. Struct. Biol.* **6**, 762-774

Topley, E.L., Davison, S., Leat, N. & Benjeddou, M. (2005). Detection of three honeybee viruses simultaneously by a single multiplex reverse transcriptase PCR. *African Journal of Biotechnology* **4**, 763-767

Tsai, Y.L., Tran, B., Sangermano, L.R. & Palmer, C.J. (1994). Detection of poliovirus, hepatitis A virus and rotavirus from sewage and ocean water by triplex reverse transcriptase PCR. *Appl. Environ. Microb.* **60**, 2400-2407.

van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. & Wickner, R.B. (editors) (2000). *Virus Taxonomy. Seventh Report of the International Committee of Taxonomy of viruses.* San Diego: Academic Press.

Weiland, J.J. & Dreher, T.W. (1989). Infectious TYMV RNA from cloned cDNA: Effects *in vitro* and *in vivo* of point substitutions in the initiation codons of two extensively overlapping ORFs. *Nucleic Acids Res.* **17**, 4675-4687.

Wiegers, F.P. (1988). Transmission of honeybee viruses by *Varroa jacobsoni* Oud. In *European research on varroatosis control*, pp. 99-104. Edited by R. Cavalloro. Commission of the European Communities: Rotterdam.

Williams, I.H. (1994). The dependence of crop production within the European Union on pollination by honeybees. *Agr. Sci. Rev.* **6**, 229-257.

Williams, K., Blake, S., Sweeney, A., Singer, J.T. & Nicholson, B.L. (1999). Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. *J. Clin. Microbiol.* **37**, 4139-4141.

Wilson, J.E., Powell, M.J., Hoover, S.E. & Sarnow, P. (2000). Naturally occurring dicistronic *cricket paralysis virus* RNA is regulated by two internal ribosome entry sites. *Mol. Cell. Biol.* **20**, 4990–4999.

Wilson, L.E., Wilkinson, S.A., Marlow, R.D., Possee, R.D. & King, L.A. (1997). Identification of recombinant baculovirus using green fluorescent protein as a selectable marker. *Biotechniques* **22**, 678-681.

Woolaway, K.E., Lazaridis, K., Belsham, G. J., Carter, M. J. & Roberts, L. O. (2001). The 5' UTR of *Rhopalosiphum padi* virus (RhPV) contains an internal ribosome entry site (IRES) which functions efficiently in mammalian, insect and plant translation systems. *J. Virol.* **75**, 10244–10249.

Yao, J.S., Strauss, E.G. and Strauss, J.H. (1996). Interactions between PE2. E1 and 6K required for assembly of Alphaviruses studied with chimeric viruses. *J. Virol.* **70**, 7910-7920.