The development of a laser microcapture method for isolating single infectious nucleopolyhedrovirus occlusion bodies from suspensions

by

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DECLARATION

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ABSTRACT

Single occlusion body (OB) infection studies provide crucial insight into the virulence and transmission of nucleopolyhedroviruses (NPVs). Current methods of NPV infection are based on end-point dilution and are therefore subject to dose errors. This study aimed to develop a laser capture microdissection (LCM) technique to isolate single infectious NPV OBs from suspensions. Helicoverpa armigera nucleopolyhedrovirus (HearNPV) OB suspensions were spotted onto membrane slides with an erioglaucine solution, which was found to increase the contrast of the OBs. When viewed using a light microscope, the OBs appeared to be round, with an average size of 1.10 μm (standard error of the mean = 0.11 μm) and were characterised by a dark solid outline. Single OBs were laser catapulted into the caps of 0.5 ml PCR tubes. Post-microdissection examination of the caps revealed the presence of an excision containing a single OB. To assess the *in vivo* infectious properties of single LCM-isolated OBs, the frequency of lethal infection with single OBs was assessed using second instar *Helicoverpa armigera* larvae and was found to be 4.83%. To determine whether the LCM method of infection follows a typical dose-mortality response, the slope of the probit-log dose regression line obtained was compared to that of droplet feeding and diet contamination. The slopes were not significantly different for the different bioassay methods, indicating that LCM does not affect the infectious properties of the OBs. To assess the suitability of DNA extracted from LCM-isolated OBs for downstream amplification, PCR amplification was conducted using DNA extracted from 1, 10 or 100 OBs. Resolution of the PCR amplicons of a core baculovirus gene, *me53*, indicated that the gene was successfully amplified in two of the eight single OB samples, and all of the samples where DNA was extracted from larger numbers of isolated OBs. This indicated that LCM had no inhibitory effects on DNA extraction or amplification and that amplification failure in the remaining single OB samples was likely a result of the concentration of template DNA being below the amplification limit. The LCM method developed in this study provides empirical evidence of the number of OBs isolated and could therefore be used to facilitate highly precise single OB studies.

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LIST OF ABBREVIATIONS

AcMNPV	Autographa californica multiple nucleopolyhedrovirus
BV	Budded virion
DNA	Deoxyribonucleic acid
DGGE	Denaturing gradient gel electrophoresis
EBs	Elementary bodies
F	Furin
FACS	Flow cytometry and fluorescence activated cell sorting
GAM	Gravity-assisted microdissection
GV	Granulovirus
HearNPV	Helicoverpa armigera nucleopolyhedrovirus
ICTV	International Committee on Taxonomy of Viruses
IR	Infrared
IPM	Integrated Pest Management
LCM	Laser capture microdissection
LD	Lethal dose
LMPC	Laser microdissection and pressure catapulting
LPC	Laser pressure catapulting
MNPV	Multiple nucleopolyhedrovirus
MOI	Multiplicity of infection
NPV	Nucleopolyhedrovirus
OB	Occlusion body
ODV	Occlusion derived virion
PEN	Polyethylene naphthalate
PET	Polyethylene tetraphthalate
PFU	Plaque forming units
PCR	Polymerase chain reaction
RENs	Restriction endonucleases
RFLPs	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
sdH_2O	Sterile distilled water

SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
Sem	Standard error of the mean
SNPV	Single nucleopolyhedrovirus
UV	Ultraviolet

CHAPTER ONE

General Introduction

1.1 *Helicoverpa armigera* pest status

Several lepidopteran species are known for the devastating impact that they have as agricultural and forest pests worldwide (Bloem *et al*., 2005). An increase in the effects of climate change has been shown to influence the distribution of phytophagous pests, with special reference being given to Lepidoptera (Thomson *et al*., 2010). Members of the *Helicoverpa* genus include some of the most damaging and highly polyphagous agricultural pests (Cunningham and Zalucki, 2014). *H. armigera* (Hübner) (Lepidoptera: Noctuidae) is one such pest and is considered to be the most widespread species of *Helicoverpa* (Jones *et al*., 2019).

H. armigera, like other Lepidoptera, are holometabolous and therefore undergo four life stages consisting of egg, larva, pupa and moth stages (Hossler, 2010). The females lay eggs on a variety of host crops including several economically important crops such as cotton, maize, sorghum and soybean (Fitt, 1989). While larvae may use the leaves as a source of nutrition, they prefer the fruit and flowering structures of the plant and hence are commonly referred to as the budworm, earworm or bollworm (Kriticos *et al*., 2015; Liu *et al*., 2010; Zalucki *et al*., 1986). Larvae usually develop through 5-6 instars, with the entire life cycle being carried out in 35-40 days (Allsopp *et al*., 2012; Vinutha *et al*., 2013). The extent of damage caused is dependent on the abundance of moths, the number of eggs they lay and the amount of larvae which reach larger, more destructive instars (Kriticos *et al*., 2015).

The significant impact that *H. arimgera* has as a pest is attributed to its polyphagous nature, high fecundity and short generation time, feeding preference for the fruiting and flowering structures of host plants, high migration ability and resistance to chemical insecticides (Cherry *et al*., 2003). *H. armigera* is a pest of numerous

agricultural, horticultural and floricultural crops and has been found in 68 plant host families worldwide, 14 of which are present in all geographical regions (Cunningham and Zalucki, 2014). While this pest originated in the Old World, international trade and long-distance migration has increased its invasion potential into the New World (Farrow and Duly, 1987; Tay *et al*., 2017). This has been highlighted through its detection in Brazil, Paraguay and Argentina, marking its entry into the New World (Kriticos *et al*., 2015). Recent studies have found that *H. armigera* is present in Central America, indicating that North America is at a high risk of invasion (Kriticos *et al*., 2015).

1.2 Control methods for *H. armigera*

1.2.1 Chemical insecticides

For centuries, several chemical pesticides have been used to control crop damage caused by *H. armigera*. This includes the use of organophosphates, carbamates and pyrethroids (Aktar *et al*., 2009). While there was an initial decline in the number of pests, continuous exposure has led to insecticide resistance (Kranthi *et al*., 2002; Mironidis *et al*., 2013). Furthermore, the high genetic diversity and long-distance migration ability of *H. armigera* increases the possibility of transfer of resistance alleles into populations which have had little exposure to these insecticides (Jones *et al*., 2019; Leite *et al*., 2014). Therefore, the insecticides were often replaced by new, more toxic chemicals. In addition to resistance, chemical insecticides have several negative effects on the environment, humans and beneficial organisms (Aktar *et al*., 2009). These effects have a greater impact on developing countries who rely on the use of older, non-patented, more toxic, environmentally persistent and low cost chemical insecticides to increase the crop yield for subsistence farming and global trade (Ecobichon, 2001).

1.2.2 Biological control as a part of Integrated Pest Management

Control of *H. armigera* is the focal point of numerous Integrated Pest Management (IPM) programs. This refers to a model designed for sustainable pest control, which aims to reduce or eradicate the use of chemical pesticides (Haase *et al*., 2015). IPM principles are based on the use of a combination of methods, including biological, physical, cultural and chemical, to reduce and maintain pest populations below the economic injury level (Jacobsen, 1997; Pedigo, 1986). Biological control agents form a crucial component of IPM strategies and have been used for the control of arthropod pests in developing and developed countries (Grzywacz *et al*., 2014). Biological control relies on the use of natural enemies, including predators, pathogens and parasitoids, to manage pest populations (Prasad and Srivastava, 2016). Particular attention has been given to entomopathogens, including various types of bacteria, fungi, nematodes and viruses (Lacey *et al*., 2015), as they are less hazardous to humans and beneficial organisms due to their high specificity and the reduction in the amount of pesticide residues found in food and the environment (Fathipour and Sedaratian, 2013). Furthermore, the use of biological control agents is more sustainable as they minimise the development of resistance (van Lenteren *et al*., 2018).

1.3 Baculoviruses

Several insect viruses have been associated with epizootics and are believed to facilitate control of insect populations in nature (Sun and Peng, 2007). This comprises RNA viruses such as tetravirus, nodavirus, cypovirus and dicistrovirus as well as DNA viruses including hytrosavirus, nudivirus, densovirus, iflavirus, entomopoxvirus, iridovirus, ascovirus and baculovirus (Prasad and Srivastava, 2016). Baculovirus-based bioinsecticides have proven to be highly efficient for the control of numerous lepidopteran pests, including *H. armigera* (Moscardi, 1999). These viruses are known for their narrow host range and low impact on beneficial insects and the environment (Gröner, 1990). Furthermore, the structure of these

viruses enable environmental persistence (Passarelli, 2011), indicating the potential use of baculoviruses for long term control strategies.

1.3.1 General characteristics

Baculoviruses are DNA viruses, which form part of the *Baculoviridae* family (Jehle *et al*., 2006). These viruses contain a double stranded circular genome, which ranges from 80-180 kbp in size (Jehle *et al*., 2006). While the genome encodes for approximately 100-160 open reading frames, there are 30 core genes which have been conserved (Herniou and Jehle, 2007; Miele *et al*., 2011). *Baculoviridae* comprises four genera, namely *Alphabaculovirus, Betabaculovirus, Gammabaculovirus* and *Deltabaculovirus* (Jehle *et al*., 2006). However, *Alphabaculovirus* and *Betabaculovirus* are the most prominent as they represent lepidopteran-specific nucleopolyhedroviruses (NPVs) and lepidopteran-specific granuloviruses (GVs), respectively (Jehle *et al*., 2006). The naming of baculoviruses has followed binomial nomenclature, where the isolate of the virus is named after the insect host from which it was isolated (Prasad and Srivastava, 2016). According to the International Committee on Taxonomy of Viruses (ICTV), names of virus species are italicised whereas virus isolates reported by the ICTV are non-italicised e.g. *Autographa californica multiple nucleopolyhedrovirus* refers to the species while Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the name of the virus responsible for infection. Baculoviruses produce proteinaceous occlusion bodies which act as the primary infectious unit. This is the distinguishing factor between NPVs and GVs as NPVs produce polyhedral OBs, with a size range of 0.4-2.5 μm, while GVs produce ovoid granules with a smaller size range of 120-350 nm × 300-500 nm (Harrison and Hoover, 2012). NPV and GV OBs are the key component of baculovirus-based insecticides as these sturdy particles enable easy formulation and application and can be stored for long periods of time (Lacey *et al*., 2015). NPVs have proven to be highly efficient for the control of several lepidopteran pests, including *H. armigera* (Moscardi, 1999).

1.3.2 Infection cycle

NPVs undergo a biphasic infection cycle, which is initiated by two distinct virion types. Although there are other routes of transmission, primary infection usually begins when an insect larva consumes food contaminated with OBs (Mazzone, 1985). Once the OBs reach the midgut, the combined action of the alkaline pH, digestive enzymes and OB-associated proteases result in the dissolution of the proteinaceous matrix, releasing the occlusion derived virions (ODVs) (Prasad and Srivastava, 2016). The ODVs pass through the peritrophic membrane, which lines the midgut, enabling direct fusion with the microvilli of the epithelial cells (Haase *et al*., 2015). The nucleocapsids enter the cytoplasm and are transported to the nucleus, via actin polymerization (Ohkawa *et al*., 2010), where the nucleocapsids are uncoated, resulting in the release of the viral DNA (Ikeda *et al*., 2015). Expression of genes occur in a sequential manner and are divided into two categories, namely early and late genes (Harrison and Hoover, 2012). Early genes are transcribed by host RNA polymerase II, followed by replication of genomic DNA and transcription of late genes, by an RNA polymerase encoded by the virus (Fuchs *et al*., 1983; Guarino *et al*., 1998). A virogenic stroma is formed, resulting in the production of progeny nucleocapsids, which leave the nucleus and enter the cytoplasm (Prasad and Srivastava, 2016). The progeny nucleocapsids as well as a portion of nucleocapsids released from the ODVs bud through the plasma membrane forming budded virions (BVs) (Harrison and Hoover, 2012).

Secondary infection is carried out by BVs, which bind to cell-surface receptors, enabling internalisation through clathrin-mediated endocytosis (Long *et al*., 2006). The endosomes are acidified by proton pumps within the endosome membrane, triggering fusion between the membrane and envelope protein, releasing the nucleocapsids into the cytoplasm (Blissard and Theilmann, 2018). The cytoplasmic nucleocapsids initiate infection within the nucleus or bud through the plasma membrane creating further progeny BVs. The midgut is not an ideal environment for baculovirus infection as cells are regularly sloughed away (Engelhard and Volkman, 1995) and are susceptible to apoptosis (Uwo *et al*., 2002). Therefore, the

BVs move to other tissues within the host including the fat body, tracheoblasts and hemocytes (Slack and Arif, 2006). In the later stages of infection, nucleocapsids are enveloped, to form ODVs which are then occluded, with polyhedrin, to form OBs (Haase *et al*., 2015). The end of the infection cycle is characterised by the release of the OBs into the environment due to host cell lysis, tissue liquefaction and rupturing of the cuticle (Harrison and Hoover, 2012).

1.3.3 NPVs

The *Alphabaculovirus* lineage has been further divided into Group I and Group II (Herniou *et al*., 2003), where Group I NPVs contain a set of approximately 12 genes, which are not found in Group II, with the most notable being the gene encoding the GP64 envelope fusion protein (Rohrmann, 2014). In contrast, the more genetically diverse Group II NPVs, which lack *gp64,* seem to utilise the Furin (F) fusion protein (Westenberg *et al*., 2002). NPV OBs contain multiple nucleocapsids, which vary in arrangement as there may be single (SNPVs) or multiple (MNPVs) nucleocapsids per an envelope (Rohrmann, 2014). MNPVs contain from 1-15 nucleocapsids per envelope, with bundles of 5-15 being the most predominant (Rohrmann, 2014). The presence of multiple nucleocapsids within SNPV and MNPV OBs enables transmission of multiple virions, resulting in an increase in the multiplicity of infection (MOI) (Sanjuán, 2017). Helicoverpa armigera nucleopolyhedrovirus (HearNPV) is an example of a SNPV, which has proven to be highly effective for the control of *H. armigera* (Moore *et al*., 2004), and therefore forms a crucial component of several biopesticides (Hatting *et al*., 2019).

1.3.4 Diversity

Baculoviruses need to overcome numerous host defence mechanisms to establish infection (Sparks *et al*., 2008). It has been suggested that virulence is dependent on the mode of viral transmission, with vertical transmission being associated with low virulence and horizontal transmission with high virulence (Stewart *et al*., 2005).

Transmission is a key factor in determining the overall fitness of a virus and relies on the variants within a population (Wargo and Kurath, 2012). While a high degree of genetic diversity has been found in baculovirus species isolated from geographically distinct host populations (Graham *et al*., 2004) and individuals within a host population (Baillie and Bouwer, 2012a; Cory *et al*., 2005), the diversity within an NPV OB is unknown.

1.4 Minimum lethal dose

Dose-response relationships, by use of bioassays, provide insight into the virulence of a NPV isolate as such experiments provide an indication of the link between exposure to a pathogen individual and the probability that lethal infection will occur. These experiments are often used to determine the minimum lethal dose or median lethal dose (LD_{50}) of an isolate (Ferrelli *et al*., 2016; Figueiredo *et al*., 1999; Victor *et al*., 2019). Several studies indicate that the number of OBs required initiate lethal infection is low (Figueiredo *et al*., 1999) and often a result of the action of a single OB (Baillie and Bouwer, 2013; Clavijo *et al*., 2010). To determine the minimum lethal dose, these studies employ one of two widely used approaches: analysis of the dose-response curve or detection of variation post-passage using theoretically low doses.

Plaque assays have been used to obtain measurements of virulence, where the ability of variants to replicate in cells in tissue culture is measured by evaluating the number of plaque forming units (PFUs) formed (Wargo and Kurath, 2012). Clem and Miller (1993) found that infection with AcMNPV resulted in an LD_{50} between 1 and 10 PFU per larva. However, each PFU may not comprise a biological clone as it may contain numerous variants (Combe *et al*., 2015). PFU counts are often inconclusive as low values, such as 0.01 PFU, as obtained by Spieker *et al*. (1996), cannot be interpreted as at least one virion would be required to initiate infection. While these studies may provide an indication of the ability of virions to initiate infection, the findings are limited to BVs in tissue culture and may not be applicable to *in vivo* studies.

In vivo studies conducted by Figueiredo *et al.* (1999) compared the lethal dose of two Spanish isolates of HearNPV (HearNPV-SP1 and HearNPV-SP2) to a Russian isolate (HearNPV-RU), using third instar *H. armigera* larvae, infected with leaf discs contaminated with the virus. The fitted regression lines revealed that 3, 6 and 7 OBs from the respective isolates could theoretically induce lethal infection in a few larvae. Bianchi *et al.* (2000) and Ferrelli *et al.* (2016) reported extremely low LD_{50} estimates of 2.9 OBs and 2 OBs, respectively, suggesting that the NPV strains described were highly virulent. The minimum lethal dose of such studies cannot be interpreted as such low estimates suggest that a fraction of an OB could initiate lethal infection. Dose-response curves do not provide a good estimate of the minimum lethal dose as the standard curve produced is sigmoidal in shape (Cory and Bishop, 1997), with minimum lethal dose values frequently occurring in the nonlinear region of the curve.

The results of several studies indicate that exposure to a single OB may result in lethal infection, where infection with a single OB is characterised by low percentage mortality (Arrizubieta *et al.*, 2015; Baillie and Bouwer, 2013; Clavijo *et al.*, 2010; Zwart *et al.*, 2009). The effects of genetic drift are considered to be strong when viral doses are low, such as that associated with a genetic bottleneck (Li and Roossinck, 2004). Therefore, low-dose infections are expected to result in detection of clones of variants, which match the genetic composition of OBs used for infection. This logic was applied in a study conducted by Smith and Crook (1988), where fourth instar *Lymantria dispar* larvae were infected with an isolate of Lymantria dispar MNPV, using the droplet feeding method. The authors found that a single genotype, which was present as a small component of the inoculum, emerged as the main progeny genotype after a single passage *in vivo*. These findings suggest that mortality occurred as a result of infection with a single OB. However, the results are limited as restriction endonucleases (RENs) were used to detect genetic variation. This technique lacks sensitivity as the RENs detect site specific restriction fragment length polymorphisms (RFLPs) and therefore does not account for random variation throughout the genome (Baillie and Bouwer, 2012a).

Furthermore, a small sample size was used, and the 17% mortality obtained is a relatively high value to be classified as a “low percentage”.

To provide a better indication the frequency of lethal infection with a single OB, lower percentages (5-10%) were used as an approximation, with 5% being the most common (Arrizubieta *et al*., 2015; Clavijo *et al*., 2010; Redman *et al*., 2016). In a study conducted by Clavijo *et al*. (2010), second instar *Spodoptera frugiperda* larvae infected, using the droplet feeding method, with a Nicaraguan isolate of Spodoptera frugiperda MNPV were found to have a LD_5 of 0.046 OBs per larva. While this value indicates the minimum lethal dose is low, the value does not correspond to a whole number indicating that the precision of the assay is questionable.

Dose-response experiments depend on several factors including temperature, choice of larval instar and method of inoculation (Cory and Bishop, 1997). While numerous bioassay methods have been developed, the majority are based on variations of droplet feeding, diet surface contamination or diet incorporation bioassays (Hughes and Wood, 1981; Ignoffo, 1965). The underlying principle of all three methods is based on end-point dilution of the sample, where the OBs in suspension are assumed to follow the Poisson distribution model (Roberts and Coote, 1965). Due to the statistical nature of end-point dilution, it is estimated that only a proportion of larvae will consume a single OB as there is a chance that a larva will receive no OB or more than one OB (Clavijo *et al*., 2010). While the findings of numerous studies support the idea of low frequency lethal infections initiated by single OBs (Arrizubieta *et al*., 2015; Baillie and Bouwer, 2013; Clavijo *et al*., 2010), the methods of inoculation are restricted as there is no empirical evidence of isolation.

1.5 Controlled virus isolation methods

Advancements in technology have enabled the modification of various single cell isolation techniques to allow for controlled isolation of single virus particles.

Gaudin and Barteneva (2015) used flow virometry, a derivative of flow cytometry and fluorescence activated cell sorting (FACS), to characterise and isolate small lipid vesicles produced by Junin virus, where isolated particles remained infectious (as per plaque assay) post-isolation. A microfluidics approach was used by Stiefel *et al*. (2012) to isolate and infect HeLa cells with single vaccinia virions, by placing the virions on the surface of the cells. While virus particles isolated using FACS and microfluidics remain infectious post-isolation *in vitro*, both techniques have not been used to isolate single OBs and the use of the techniques is restricted to inoculation of cell or tissue culture and may not suitable for larval consumption.

Micromanipulation enables label-free isolation as microscopically visualised particles are manually selected, using microneedles or micropipettes made of ultrathin glass capillaries (Bakoss, 1970; Sherman, 1973). Rivkin *et al*. (2006) used this technique to inoculate fourth instar *Spodoptera littoralis* larvae with recombinant AcMNPV OBs. Inoculation with OBs, isolated using micromanipulation, is carried out using microinjection, which is often problematic due to the side effects of the anaesthesia and injury at the site of injection (Klein, 1978). This not only has a higher impact on smaller instars but also fails to account for oral infectivity (Ikeda *et al*., 2015). The size of the particles obtained are often limited as the microcapillaries used typically contain an inner diameter of 2-10 µm (Lasken *et al*., 2005). Furthermore, aspiration of particles in solution could result in contamination with adjacent particles or particles could adhere to the inner surface of the microcapillary used. As such, current virus isolation methods are not suitable for isolation of single NPV OBs for subsequent infection studies.

1.6 LCM

Laser capture microdissection (LCM) was developed by the National Cancer Institute of the National Institutes of Health in Bethesda, to isolate microscopically visualised cells from histological tissue sections of solid tumours, for downstream molecular analysis (Emmert-Buck *et al*., 1996). Although the technique has become popular in various fields of biology, the use of LCM to study microorganisms is

limited. The majority of microorganism based studies have exploited LCM to determine plant-nematode, plant-bacterium and plant-fungi interactions by means of gene expression analysis (Gomez and Harrison, 2009; Ramsay *et al*., 2006). In a study conducted by Kang *et al*. (2011), LCM was used to isolate single bacterial cells, from which transcripts were successfully amplified for transcriptome analysis. The use of the technique has not only led to advances in molecular characterisation but has also enabled isolation of live cells for subsequent infection. In one such study, LCM was used to isolate HeLa cells, which were infected with chlamydial elementary bodies (EBs) at a low MOI (Podgorny *et al*., 2015). Single cells, which were assumed to be infected by a single *Chlamydia trachomatis* inclusion, were harvested and used for infection in cell culture. Maturation of the inclusions led to the release of EBs and infection in the host cell monolayer (Podgorny *et al*., 2019), indicating that single inclusion derived *Chlamydia* could cause infection in tissue culture. While LCM has not been used to isolate single virus particles, the above mentioned studies indicate that the technique is highly versatile and could be promising for the isolation of single NPV OBs.

1.6.1 Infrared LCM

The initial system used by Emmert-Buck *et al*. (1996) relied on the use of an infrared (IR) laser and was later commercialised by Arcturus Engineering as the PixCell system. This system is based on the use of a specialised cap containing a thin, transparent thermoplastic film, which is placed over a standard glass slide containing the sample of interest (Vandewoestyne *et al*., 2013). The sample is visualised microscopically and selected cells adhere to the film as the result of a fixed-position, short duration, focused pulse from an IR laser (Emmert-Buck *et al*., 1996). The pulse from the laser causes localised melting of the thermoplastic film (Rodriguez-Canales *et al*., 2013). The force of adhesion between the cells and the film is greater than that of the glass slide, enabling isolation of specific cells (von Eggeling *et al*., 2007). The cells are obtained by removing the cap and transferring it to a microcentrifuge tube for further processing (Fend and Raffeld, 2000). Since IR LCM systems are dependent on physical contact for isolation of cells, samples

are at risk of contamination (Ahmed, 2006). Furthermore, this system is known to generate transient heat (90 °C), which could negatively affect nucleic acids (Goldstein *et al*., 1998) and compromise the infectious properties of the particles.

1.6.2 Ultraviolet LCM

Gravity-assisted microdissection (GAM) and laser microdissection and pressure catapulting (LMPC) systems rely on the use of ultraviolet (UV) lasers. UV LCM systems are based on the use of polymer membranes, polyethylene naphthalate (PEN) or polyethylene tetraphthalate (PET), on which the samples are loaded (Vogel *et al*., 2007). Microdissection is carried out as the UV-laser emits photon energy (linear absorption) resulting in plasma formation (Vogel *et al*., 2007). Thermal expansion of the plasma is accompanied by rapid, adiabatic cooling, therefore, adjacent matter is unaffected as the time available for heat transfer is < 1 µs (Vogel *et al*., 2007). This phenomenon is known as "cold ablation" (Burgemeister, 2005). After dissection, LMPC systems, such as the PALM Microbeam system (PALM Zeiss Microlaser Technologies, Munich, Germany), use more powerful pulses of a defocused laser beam, which causes photonic pressure and catapults isolated cells into a collection tube above the sample (Schüitze and Lahr, 1998). In contrast, GAM systems, such as that developed by Leica (Leica Microsystem, Wetzlar, Germany), rely on gravity to collect microdissected cells from an inversely mounted slide (Rodriguez-Canales *et al*., 2013). The use of LMPC and GAM systems enable non-contact isolation of cells, thereby minimising the risk of contamination (Gross *et al*., 2015).

1.7 Study justification

The results of several studies indicate that a single OB is capable of initiating low frequencies of lethal infection (Arrizubieta *et al*., 2015; Baillie and Bouwer, 2013; Clavijo *et al*., 2010). These studies are based on bioassays conducted using droplet feeding, diet surface contamination or diet incorporation (Hughes and Wood, 1981; Ignoffo, 1965), where inoculation with a single OB is based on end-point dilution. These methods of inoculation rely on the assumption that the OBs in suspension follow the Poisson distribution model and are therefore ingested at random and do not form clumps (Roberts and Coote, 1965). However, due to the statistical nature of end-point dilution (Gross *et al*., 2015), there is no empirical evidence of isolation of single OBs. As such, it is expected that only a fraction of larvae will be exposed to a single OB, with the rest receiving no OB or more than one OB (Clavijo *et al*., 2010). In a study conducted by Clavijo *et al*. (2010), larvae infected with a theoretical dose of 1 OB were found to have a LD_5 of 0.046 OBs, suggesting that current methods of inoculation are subject to dose-errors due to the nature of end-point dilution.

In addition to minimum lethal dose studies, single OB infections play a crucial role in studies, which aim to determine the level of genetic variation in a single OB (Arrizubieta *et al*., 2015; Clavijo *et al*., 2010), highlighting the need for a precise method of isolation of these particles. Current virus isolation methods are not suitable as methods, such as FACS and microfluidics, require sample preparation with fluorescent dyes or antibodies (Hu *et al*., 2016; Lippé, 2017) and are limited to cell or tissue culture while label-free micromanipulation approaches lack the sensitivity required to isolate single NPV OBs.

LCM has proven to be a highly versatile technique shown by its ability to isolate single cells or groups of cells from fixed or frozen samples as well as live cultures (Burgemeister, 2005; Stich *et al*., 2003). Newer systems, which encompass the use of a UV laser, enable contact-free isolation of cells, which are typically unaffected by the heat of the laser (Gross *et al*., 2015; Vogel *et al*., 2007). While the technique

has not been used to isolate single virus particles, the use of LCM to isolate single cells for subsequent infection *in vitro* (Podgorny *et al*., 2019) suggests that a laser microcapture method could be suitable for the isolation of single NPV OBs for subsequent infection *in vivo*. Due to the high efficiency of HearNPV for the control of *H. armigera* (Moore *et al*., 2004), extensive research has been conducted on the virulence and diversity of the virus (Baillie and Bouwer, 2012a; Ogembo *et al*., 2005). As such, HearNPV was used as a model NPV for the development of the technique.

1.8 Objectives and aims

1.8.1 Main objective

To develop a laser microcapture method that allows infectious NPV OBs to be isolated from suspensions, using HearNPV as a model NPV.

1.8.2 Specific aims

- To develop a LCM technique for the isolation of single NPV OBs from suspensions.
- To assess the *in vivo* infectious properties of OBs isolated using LCM.
- To determine whether it is possible to extract and amplify DNA from single LCM-isolated OBs.

1.9 Dissertation outline

This dissertation consists of three research chapters (Chapters 2, 3 and 4), as well as a General Introduction chapter (Chapter 1) and a General Discussion and Conclusion chapter (Chapter 5). All research chapters have been written in the format of scientific papers and the repetition of information on certain aspects is unfortunately unavoidable in this format. Abbreviations used throughout the dissertation are introduced in each chapter. The first chapter in this dissertation contains the rationale and motivation for the study, the objectives and aims of the research, as well as a general introduction on the importance of developing a suitable method for the isolation of single NPV OBs from suspensions. Chapter 2 investigates the use of LCM for the isolation of NPV OBs from suspensions. Chapter 3 assesses the *in vivo* infectious properties of single NPV OBs isolated using LCM. Chapter 4 assesses the suitability of DNA extracted from single LCM-isolated OBs for downstream amplification. Chapter 5 provides an overview of Chapters 2, 3, and 4, integrates the results from these chapters and suggests potential research avenues that may be pursued on the basis of the findings.

CHAPTER TWO

The development of a laser capture microdissection technique for the isolation of single nucleopolyhedrovirus occlusion bodies from suspensions

2.1 Abstract

Current nucleopolyhedrovirus (NPV) transmission studies employ end-point dilution to isolate single occlusion bodies (OBs) and are therefore based on statistical models. This study aimed to develop a laser capture microdissection (LCM) method for the isolation of single NPV OBs from suspensions. Helicoverpa armigera nucleopolyhedrovirus (HearNPV) OB suspensions were spotted onto membrane slides with erioglaucine, which was found to increase the contrast of these particles. Under a light microscope, the OBs appeared to be round, with an average size of 1.10 µm (sem = 0.11 µm) and were characterised by a dark solid outline. Single OBs were laser catapulted into the caps of 0.5 ml PCR tubes. Examination of the caps post-microdissection revealed the presence of a membrane cut out containing a single OB. These findings indicate that a LCM technique was successfully developed to isolate single NPV OBs from suspensions. The LCM technique provides empirical evidence of the number of OBs isolated and could therefore be used to facilitate highly precise single OB studies.

2.2 Introduction

NPVs are a group of insect pathogenic viruses, which have proven to be highly efficient for the control of numerous lepidopteran pests (Lacey *et al*., 2015). These viruses are members of the *Alphabaculovirus* genus and are known for the production of large, polyhedral OBs, which comprise several occlusion derived virions (Jehle *et al*., 2006). As such, the structure of NPV OBs facilitate the collective spread of numerous virions during horizontal transmission (Sanjuán, 2018). Therefore, the overall fitness of the virus depends on the variants present within an OB (Wargo and Kurath, 2012).

Current transmission studies often employ end-point dilution to isolate single OBs. In one such study, the genetic diversity within an OB was assessed by infecting larvae with single OBs and detecting variation post-passage (Clavijo *et al*., 2010). Infection resulted in a LD_5 estimate of 0.046 OBs per larva. While this value indicates that the frequency of infection is low, the value suggests that a fraction of an OB caused infection. Due to the statistical nature of the end-point dilution method (Gross *et al*., 2015; Roberts and Coote, 1965), the results of such studies are limited to the presumption of ingestion of a single OB.

Therefore, a method which provides empirical evidence of isolation of single OBs would enable major advancements in the field of baculovirology. A few single cell isolation techniques have been adapted to isolate single virus particles. Flow cytometry and fluorescence activated cell sorting (FACS) as well as microfluidic techniques require preparation of samples prior to isolation. This includes labelling of virus particles with fluorescent dyes or antibodies (Hu *et al*., 2016; Lippé, 2017), which could affect the properties of the particles during downstream applications.

While label-free isolation can be carried out using micromanipulation, particles are isolated using glass micropipettes thereby restricting the size of the particles to the diameter of the microcapillaries (Lasken *et al*., 2005). Furthermore, the particles are aspirated in solution, which could result in the uptake of adjacent particles or

adherence to the inner surface of the microcapillary use. The use of current virus isolation techniques is therefore not suitable for the isolation of NPV OBs.

LCM is a single cell isolation technique which enables isolation of microscopically visualised cells (Curran *et al*., 2000). The use of the technique has proven to be highly versatile, shown by its ability to isolate individual cells or groups of cells from fixed or frozen samples as well as live cultures (Burgemeister, 2005; Stich *et al*., 2003). While initial infrared (IR) laser-based systems, which are dependent on physical contact for isolation of single cells, are prone to contamination with adjacent cells (Ahmed, 2006), newer ultraviolet (UV) laser-based systems have enabled contact-free isolation, thereby minimising the risk of contamination (Gross *et al*., 2015). Although the technique was developed for the isolation of single cells from histological sections of solid tumours (Emmert-Buck *et al*., 1996), its use has been extended to various fields of biology.

Microbiology specific studies have used LCM isolation to determine plant-nematode, plant-bacterium and plant-fungi interactions by means of gene expression analysis (Gomez and Harrison, 2009; Ramsay *et al*., 2006). Further use of the technique was shown by its ability to isolate live bacterial cells for subsequent infection in cell culture and single cell genomic amplification (Kang *et al*., 2011; Podgorny *et al*., 2019). While LCM has been used to isolate various microorganisms, the use of the technique has not been applied to virus particles, including NPV OBs. The large size and light refractory properties of NPV OBs allow visualisation of these particles under a light microscope (Slack and Arif, 2006), suggesting that a LCM method could be suitable for the isolation of these particles. This studied aimed to develop a LCM method for the isolation of single NPV OBs from suspensions.

2.3 Materials and methods

2.3.1 Insect rearing

H. armigera laboratory cultures were established from eggs originally obtained from the Agricultural Research Council (Pretoria, South Africa). The larvae were reared on an artificial wheatgerm diet as described by Bot (1966). The moths were reared on a 5% (w/v) sucrose solution in glass jars enclosed by nets to facilitate oviposition. Eggs and pupae were treated with sodium hypochlorite solutions at 0.2% (w/v) and 0.25% (w/v), respectively, to reduce microbial contamination. All cultures were kept at a constant temperature of 25 °C (±1), relative humidity of 60% (±5) and a 12:12 h light:dark photocycle.

2.3.2 Virus propagation and purification

A HearNPV (a SNPV) isolate was propagated in third instar (± 6 d-old) *H. armigera* larvae via the diet contamination method (Evans and Shapiro, 1997). Larvae that displayed signs of HearNPV infection were harvested and frozen, prior to liquification. OBs were purified based on a modified version of the protocol described by Crook and Payne (1980). Larvae were homogenised in 0.1% (w/v) sodium dodecyl sulphate (SDS) and subjected to filtration and low speed centrifugation to eliminate large insect debris. OBs were purified using 40-65% (w/w) discontinuous sucrose gradients and centrifugation at 47 500 ×*g* for 90 min. The OB band that formed at the 55-60% interface was removed and subjected to three wash steps in sdH_2O, at 20 400 ×*g* for 30 min. The final OB pellet was resuspended in sdH_2O and stored at -20 °C. The concentration of OBs was determined using an improved Neubauer haemocytometer (Hawksley, Lancing, UK) and phase-contrast microscopy.

2.3.3 SEM

Purified OBs were examined using scanning electron microscopy (SEM) with the aid of the FEI Quanta ESEM 400 scanning electron microscope. The OB suspension was mounted directly onto an aluminium stub. The sample was left to dry at room temperature before being sputter coated with 2.5 nm platinum using an automated sputter coater (Model: Quorum Emitech K550X).

2.3.4 LCM

HearNPV suspension (6.08×10^7 OBs/ml), previously stored at -20 °C was vortexed vigorously before use. The OB suspension, containing 0.17 mg/ml erioglaucine was mounted onto polyethylene naphthalate (PEN) membrane-coated glass slides (Carl Zeiss MicroImaging) in a dropwise manner. Slides were left to dry at room temperature for 20-40 minutes before performing laser microdissections. The PALM MicroBeam IV laser-capture microdissection system, coupled with an inverted microscope (AxioObserver, Carl Zeiss), solid state pulsed 355 nm UV-A laser and PALMRobo v4.5 software, was used to isolate single OBs. Microscopic images of the OBs on the membrane slides were viewed using the brightfield setting and the video display obtained from the AxioCam ICc1 camera. OBs were selected for microdissection by outlining them on the video display of the microscopic images. A laser microbeam focused through the selected objective lens was used to isolate single OBs by cutting out the selected region of interest of the membrane, leaving a small connecting bridge to which a single laser pulse of energy (Robo LPC) was applied, which catapulted the excisions into the caps of 0.5 ml PCR tubes. All excisions were done so that the OBs were placed in the middle of a circular cut out, with a minimum area of approximately 250 μm^2 (maximum ± 450 μm^2). The parameters used for the microbeam dissections and laser pressure catapulting (LPC) are listed in Table 1. Control cut outs were obtained from slides spotted with 0.17 mg/ml erioglaucine solutions.

Table 1. Settings used to isolate and catapult single HearNPV OBs into the cap of 0.5 ml PCR tubes

Objective lens	PEN slide thickness (mm)	Microbeam		LPC	
		Cut energy	Focus	Energy	Focus
63×	1	40-43	13	60-62	7
100×	0.17	42-45	60	70-72	70

2.4 Results

2.4.1 SEM

Microscopic examination of the sample indicated that the OBs represented typical NPV OBs as they were polyhedral in shape. The size of the OBs ranged from 0.75 to 1.25 µm, with an average diameter of 1.05 µm (sem = 0.07 µm; n = 30). The OBs were irregular in shape with the majority being more spherical (Figure 1). High magnification images revealed that the surface of the OBs was rough (Figure 1, E and F).

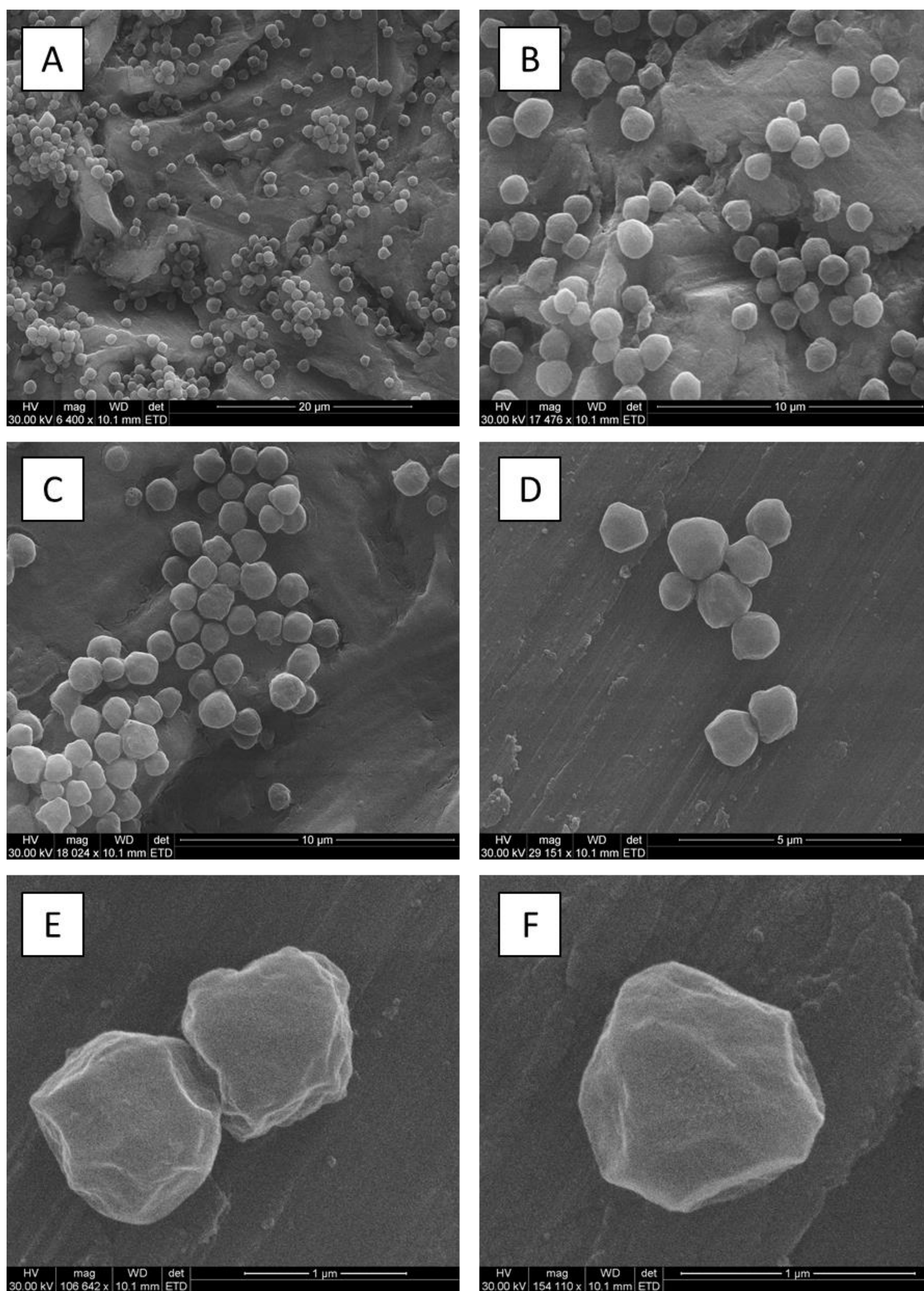

Figure 1. Scanning electron micrographs of polyhedral HearNPV OBs at low to medium magnification (**A-D**), and high magnification (**E-F**). Note the irregular shape and rough surfaces of the OBs at high magnification.

2.4.2 LCM

Analysis of the slides using the inverted microscope revealed that pores were present throughout the PEN membrane (Figure 2A). When compared to the pores, the OBs appeared to contain a dark solid outline (Figure 2B). The size of the OBs ranged from 0.81 to 1.70 µm, with an average diameter of 1.10 (sem = 0.11 µm; n = 30).

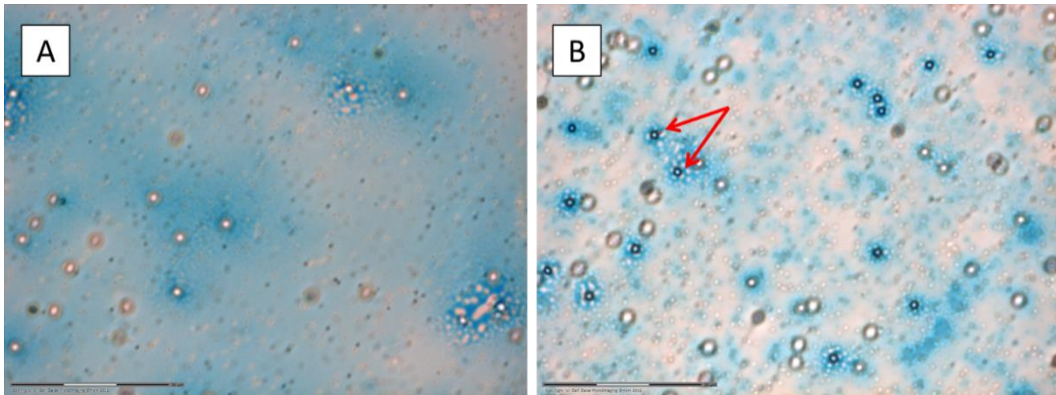

Figure 2. PEN membrane slides loaded with control suspension (**A**) and HearNPV suspension (**B**), indicating the presence of OBs (red arrows). Scale bars represent 30 µm.

Post-microdissection examination of the 0.5 ml PCR tube caps indicated that a single OB was successfully catapulted into the cap as shown by the presence of a membrane cut out containing a single OB (Figure 3).

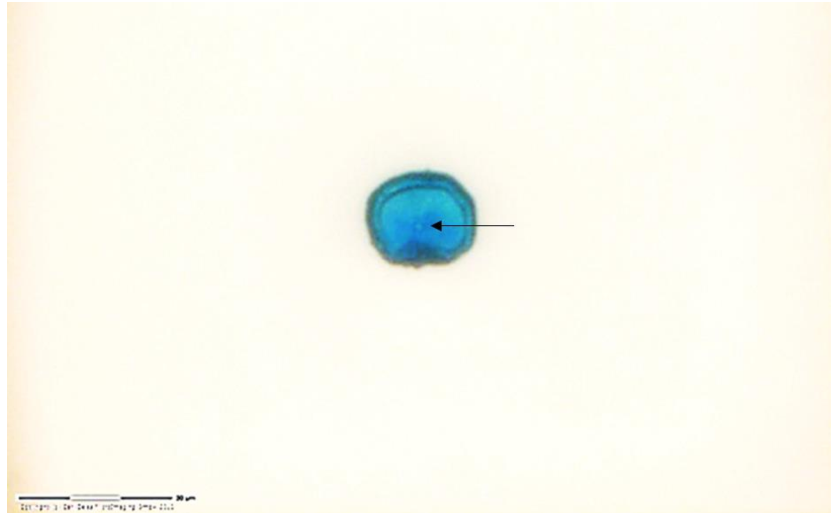

Figure 3. The cap of a 0.5 ml PCR tube containing a single OB (black arrow) attached to a PEN membrane cut out. Scale bar represents 30 µm.

2.5 Discussion

Morphological identification of the OBs, using SEM revealed that they were polyhedral in shape with a size range of 0.75 to 1.25 µm. These findings were similar to the HearNPV isolates characterised by Eroglu *et al*. (2018) and Ferrelli *et al*. (2016), where polyhedral OBs were reported to have a size range of 0.85 to 1.25 µm and 0.6 to 1.2 µm, respectively. The polyhedral OBs of a *Spodoptera exempta* nucleopolyhedrovirus isolate had a larger size range of 1.3 to 1.9 µm (Escasa *et al*., 2019), while the OBs described in this study were within the size range of a Lonomia obliqua nucleopolyhedrovirus isolate (Wolff *et al*., 2002). Comparison to previous studies indicate that the OBs in suspension belong to a NPV.

When viewed using the inverted microscope, the PEN membrane slides were found to contain circular pores with a similar size range to the OBs. While isolation is achieved by selecting particles on the video display of the live microscopic images, the presence of the pores resulted in poor quality images, making differentiation between the membrane and the OBs difficult. While densely packed OBs were easily distinguished, large gaps between the OBs were required for efficient isolation of single OBs. The PEN membrane slides on which the suspensions were mounted onto are known to be optically heterogenous, often resulting in the appearance of artificial objects (Podgorny and Lazarev, 2017), accounting for the presence of the pores in the membrane. While phase contrast microscopy is typically used to view NPV OBs (Eroglu *et al*., 2018; Kumar *et al*., 2011; Teakle *et al*., 1985), the use of this setting is known to enhance the optical heterogeneity of the membrane (Podgorny and Lazarev, 2017) and is therefore not suitable. The addition of erioglaucine, a common blue dye used to assess larval feeding (Bouwer and Avyidi, 2006), was found to increase the contrast of the OBs, making them more distinguishable at low sample concentrations. Furthermore, the dye served as a marker on the slide as the colour gave an indication of where the sample was spotted onto.

It is thought that the poor quality images also arise as a result of the thickness of the membrane slides, which are typically 1 mm, and the absence of a mounting medium and coverslip (Podgorny and Lazarev, 2017; van Dijk *et al*., 2003). When the OBs were viewed at 1000× magnification, using the 0.17 mm slide and immersion oil, it was found that the pores were still visible on the membrane, indicating that the use of the thinner membrane slides with immersion oil had little to no effect on the presence of the pores. While the use of the 100× objective, in conjunction with the thinner slides and immersion oil, resulted in the OBs being more apparent on the membrane, the OBs were still visible and distinguishable at 630× magnification.

LCM instruments, such as the PALM MicroBeam IV used in this study, are generally equipped with a stage that can house a 1 mm membrane slide. The use of the 100× objective requires a specialised stage and thinner membrane slides, which are more expensive when compared to regular glass slides (Vandewoestyne and Deforce, 2010). While visualisation of the OBs at 1000× magnification is recommended, it is not crucial as the OBs are clear at 630× magnification. As such the additional cost of the specialised stage and thinner slides could be eliminated.

The OB measurements obtained using the inverted microscope were found to be greater than the measurements obtained using SEM. This could have occurred as the inverted microscope is a light microscope and is therefore less accurate when compared to the SEM. Furthermore, the use of erioglaucine could have resulted in an increase the apparent diameter of the OBs.

The system used in this study comprised a solid state pulsed 355 nm UV-A laser, which was used to isolate single OBs. UV-damage has been noted in cells directly in line with the UV laser cutting path (Espina *et al*., 2006). Therefore, all OBs used in this study were placed in the middle of the cut out, which had a minimum area of approximately 250 μm^2. The laser used for the microbeam dissection falls within the UV-A range (320 - 400 nm) of the spectrum (Stich *et al*., 2003). Therefore, the effect of unfocused laser radiation during dissection and catapulting is expected to be minimal as the wavelength is far from the peak of absorption of DNA and protein

(Mohanty *et al*., 2002; Srinivasan, 1986). Furthermore, the final Robo LPC pulse was directed to the connecting bridge and not the centre of the excision (where the OB is typically located). Therefore, isolated OBs are expected to have uncompromised viability.

Most low dose infection studies rely on the use of inoculation methods, which are based on end-point dilution, where the probability of ingestion of a single OB is described by the Poisson distribution model (Clavijo *et al*., 2010; Huber and Hughes, 1984; Zwart *et al*., 2009). A main assumption of this distribution model is that the OBs in solution do not form clumps (Roberts and Coote, 1965). Microscopic analysis of the sample indicated the presence of aggregates at both high and low concentrations (data not shown). While OB samples are typically vortexed or subjected to sonification (Biji *et al*., 2006; Kolodny-Hirsch *et al*., 1993) to eliminate clumping, not all of the OBs dissociate resulting in the presence of aggregates irrespective of the concentration. Therefore, the OBs in solution may not always follow a Poisson distribution. The LCM technique developed in this study enables visualisation of the OBs under a light microscope. Thus, clumps of OBs can be avoided providing empirical evidence of isolation of a single OB.

In conclusion, a LCM technique was successfully developed for the isolation of single NPV OBs from suspensions. The technique can be used to facilitate highly precise single OB studies as the presence of a single OB is guaranteed.

CHAPTER THREE

Assessment of the *in vivo* infectious properties of single nucleopolyhedrovirus occlusion bodies isolated using laser capture microdissection

3.1 Abstract

The precision of low dose infection studies is restricted as traditional methods of infection are subject to dose errors. This study aimed to assess the *in vivo* infectious properties of nucleopolyhedrovirus (NPV) occlusion bodies (OBs) isolated using laser capture microdissection (LCM). The frequency of lethal infection in second instar *Helicoverpa armigera* larvae inoculated with single LCM-isolated Helicoverpa armigera nucleopolyhedrovirus (HearNPV) OBs was found to be 4.83%. To validate the use of the LCM method of inoculation, the median lethal dose (LD_{50}) of LCM-isolated OBs was estimated and compared to that of traditional bioassays, namely droplet feeding and diet contamination. Analysis of the probit-log dose regression lines revealed that the slopes were not significantly different for the various bioassay methods, indicating that assays that used LCM-isolated OBs produced a typical dose-mortality response. Comparison of the LD_{50} estimates indicated that the value obtained for the LCM method (188.7 OBs) was higher than that of droplet feeding (89.6 OBs) but lower than that of diet contamination (493.4 OBs). Due to the high precision of OB isolation using LCM, the LCM method theoretically provided the best representation of the LD_{50}. The results of this study indicate that the infectious properties of NPV OBs were not compromised during isolation. The LCM method can be used for better, empirical estimation of lethal doses *in vivo*.

3.2 Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a major agricultural pest, which originated in the Old World (Tay *et al*., 2013). Detection of *H. armigera* in South and Central America has highlighted the invasion threat posed to the New World (Kriticos *et al*., 2015). The polyphagous nature of this insect has resulted in a severe loss in yield of several economically important crops including cotton, maize, sorghum and soybean (Fitt, 1989). Efforts to control crop damage caused by these pests were reliant on the use of chemical insecticides. This has not only resulted in insect resistance (Kranthi *et al*., 2002; Mironidis *et al*., 2013), but also environmental contamination and death of non-target organisms, which are often beneficial (Aktar *et al*., 2009).

Baculoviruses have proven to be a crucial component of Integrated Pest Management (IPM) strategies (Knox *et al*., 2015). While *Baculoviridae* comprises four genera, *Alphabaculovirus, Betabaculovirus, Gammabaculovirus* and *Deltabaculovirus, Alphabaculovirus* and *Betabaculovirus* are prominent as they represent lepidopteran-specific nucleopolyhedroviruses (NPVs) and lepidopteran-specific granuloviruses (GVs), respectively (Jehle *et al*., 2006). Baculovirus virions are occluded in a crystalline protein matrix of either polyhedrin (NPVs) or granulin (GVs) forming a structure known as the occlusion body (OB) (Slack and Arif, 2006). The structure of an OB enables persistence of virion infectivity as it confers high stability under normal environmental conditions (Rohrmann, 2019). Furthermore, these viruses are known for their narrow host range and low impact on beneficial insects and the environment (Gröner, 1990).

To determine the suitability of a baculovirus strain as a potential bioinsecticide, dose-response experiments have been used to calculate the minimum number of OBs required to initiate lethal infection or to determine the median lethal dose, which along with the dose-response curve provides an indication of the virulence of the virus (Bianchi *et al*., 2000; Figueiredo *et al*., 1999; Ogembo *et al*., 2005). While a few studies suggest that the consumption of a single OB results in lethal

infection (Arrizubieta *et al.*, 2015; Clavijo *et al.*, 2010), current methods of inoculation rely on end-point dilution, which is of statistical nature (Gross *et al.*, 2015). This not only suggests that current studies may provide an inaccurate representation of the minimum lethal dose but also that there may be deviations in the LD_{50} estimates.

To the best of our knowledge, micromanipulation is the only other approach that has been used to isolate NPV OBs for subsequent *in vivo* infection thus far (Engelhard *et al.*, 1994; Rivkin *et al.*, 2006). While the use of this technique, in conjunction with microinjection, enables delivery of exact numbers of OBs to various parts of the insect, the OBs are only infectious when inoculated *per os* (Ikeda *et al.*, 2015). Furthermore, the use of microinjection is problematic due to possible side effects of anaesthesia and injury at the site of infection (Klein, 1978), which is often a greater concern when handling smaller instars.

The LCM technique has been used for a variety of applications, including the isolation of cells for subsequent infection. This was shown by Podgorny *et al.* (2015) who used LCM to isolate single HeLa cells, infected with *Chlamydia trachomatis* inclusions. The isolated cells were shown to cause infection in cell culture. While the technique has been used in a few studies in the field of microbiology, it has not been applied to the isolation of viruses, including NPV OBs for subsequent infection *in vivo*. In Chapter 2, a technique was developed for the isolation of single NPV OBs using LCM.

This study aimed to assess the *in vivo* infectious properties of LCM-isolated NPV OBs. This was done by determining the frequency of lethal infection with a single OB and obtaining a LD_{50} estimate by the use of a dose-response curve. To verify the use of the method, the slope of the dose-mortality response estimate, using the LCM method, was compared to that of traditional bioassays, namely droplet feeding and diet contamination.

3.3 Materials and Methods

3.3.1 Insect rearing

H. armigera laboratory cultures were established from eggs originally obtained from the Agricultural Research Council (Pretoria, South Africa). Cultures were maintained according to Chapter 2, section 2.3.1 (page 20).

3.3.2 Virus propagation and purification

A HearNPV (a HearSNPV) isolate was propagated in third instar (± 6 d-old) *H. armigera* larvae as described in Chapter 2, section 2.3.2 (page 20). The gradient-purified sample was vortexed vigorously before use in subsequent bioassays.

3.3.3 LCM

Individual HearNPV OBs, as viewed at 630× or 1000× magnification, were catapulted into 0.5 ml PCR tube caps containing 100 µl of artificial wheatgerm diet (Bot, 1966). The same procedure was followed for groups of OBs, which were catapulted onto the same diet plug to form the various doses. Isolation was done using the PALM MicroBeam IV laser-capture microdissection system as described in Chapter 2, section 2.3.4 (page 21). The diet within the caps, containing LCM OBs were photographed using a Zeiss SteREO Discovery.V12 Stereomicroscope.

3.3.4 Bioassays

3.3.4.1 LCM

Second instar (± 4 d-old) larvae were starved for 1-2 hours before being placed in the 0.5 ml PCR tubes containing OB cut outs described in Chapter 3, section 3.3.3 (page 33). Tubes were pierced with a total of four holes along the length of the tube, using a 1cc insulin syringe containing a 29g × ½” needle. The tubes were put in a

box with the cap facing down and moist paper towel was placed onto the conical portion of the tubes, to increase humidity and prevent drying out of the diet. Larvae that consumed the top layer of diet within 24 h were transferred back to individual rearing containers containing artificial diet. The same procedure was followed using control larvae; however, control larvae were put into 0.5 ml PCR tubes containing diet and control (no OB) cut outs. Mortality was scored seven days post infection. Eight replicates were carried out using a total of 145 larvae for single OB infections. A LD_{50} estimate was obtained using eight to twelve replicates for a range of five doses, with 30-45 larvae per dose. A total of 210 larvae were used for control infections. Bioassays were kept at a constant temperature of 25 °C (±1), relative humidity of 60% (±5) and a 12:12 h light:dark photocycle.

3.3.4.2 Diet contamination

Second instar (± 4-d old) larvae were infected using the diet surface contamination method. The HearNPV inoculum was diluted and 10 µl of the suspension was dispensed onto the surface of 100 µl of artificial wheatgerm diet filled in the caps of 0.5 ml PCR tubes. Larvae were starved for 1-2 hours before being placed in tubes, which were pierced and covered as described in section 3.3.4.1 (page 33). Larvae that consumed the diet within 24 h were transferred back to individual rearing containers containing artificial diet. The same procedure was followed using control larvae; however, control larvae were put into tubes where the surface of the diet was treated using sdH_2O. Infection was performed using eight to nine replicates for a range of five doses, with 80 larvae per dose and 100 larvae for the control. Bioassays were kept at a constant temperature of 25°C (±1), relative humidity of 60% (±5) and a 12:12 h light:dark photocycle.

5.3.4.3 Droplet feeding

Second instar (± 4-d old) *H. armigera* larvae were inoculated using a modified version of the droplet feeding method described by Hughes and Wood (1981). Larvae were starved for 2-4 h at 25 °C prior to inoculation. OB suspensions

contained 0.5 mg/ml erioglaucine (a blue dye) to detect larvae which had imbibed the solution. Larvae which had imbibed the suspension were transferred to individual rearing containers containing artificial wheatgerm diet. Infection was performed using six to nine replicates for a range of five doses, with a total of 90 larvae per dose. Control larvae (n = 94) were treated similarly, using sdH_2O. Bioassays were kept at a constant temperature of 25 °C (±1), relative humidity of 60% (±5) and a 12:12 h light:dark photocycle. Doses were calculated using the mean volume imbibed, which was determined using a modified version of the protocol described by Bouwer and Avyidi (2006). Fluorescence was measured using the JASCO FP-8200 Spectrofluorometer. This was performed using five replicates of 20 larvae (n = 100). Second instar larvae were found to have imbibed a volume of 252.2 ± 75.1 nl.

3.3.5 Data analysis

LD_{50} values of all three methods were estimated by Probit analysis (Finney, 1971). The slopes of the probit-log dose regression lines were compared using PoloPlus 2.0 software (LeOra Software, Berkeley, CA).

3.4 Results

3.4.1 Imaging of diet containing LCM OBs

To verify the presence of the OBs on the diet, the caps on which the OBs were catapulted into were microscopically examined. An increase in the number of catapulted excisions resulted in a larger area of the diet being covered by a blue zone (Figure 4). The blue zones were formed due to the accumulation of erioglaucine, from the membrane, and were restricted to the middle of cap indicating that the excisions were catapulted onto the same area.

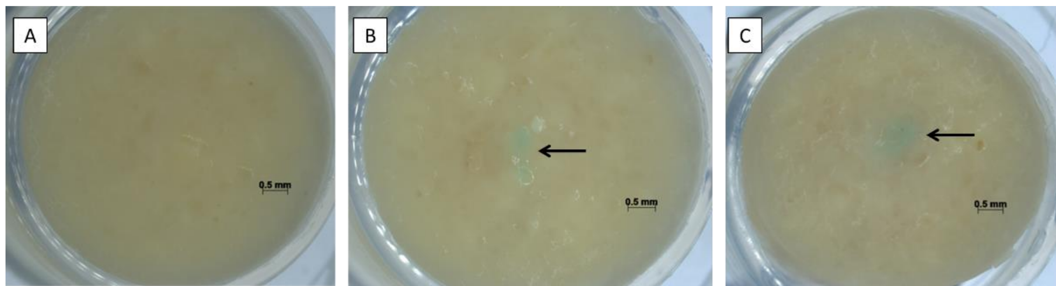

Figure 4. Caps of 0.5 ml PCR tubes showing blue zones (black arrows), formed by the dye, on the diet when different numbers of excisions were laser catapulted. **A**, 1 excision; **B**, excisions containing 200 OBs; and **C**, excisions containing 625 OBs.

3.4.2 Bioassays

When larvae were infected with single OBs, the observed frequency of lethal infection was found to be 4.83%. Lethal infection of larvae was characterised by typical symptoms associated with polyhedrosis disease. Lethal infection occurred when cut outs were made using either objective lens. No mortality was observed in larvae infected with control cut outs. The dose-mortality response obtained when second instar larvae were infected, using the LCM method, was compared to that of larvae infected using the droplet feeding method and diet contamination method (Table 2). The LD_{50} value obtained when larvae were infected using the LCM method was found to be higher than that of droplet feeding but lower than that of

diet contamination. The slopes of the probit-log dose regression lines ranged from 0.98 to 1.36, with the slope of the droplet feeding method being the lowest and the slope of the diet contamination method being the highest. The Chi-square (χ^2) value obtained for the LCM method was higher than that of either the droplet feeding or diet contamination method. The χ^2 values calculated for all three methods were less than the predicted values, indicating that all three methods provided a good fit of the probit model.

Table 2. Probit analysis of the dose-mortality response of second instar *H. armigera* larvae when inoculated with HearNPV OBs, using three different methods

Method	LD_{50} (OBs)	Slope ± SE*	χ^2
LCM	188.7 (131.3-312.4)	1.34 ± 0.23	2.43
Droplet feeding	89.6 (65.6-120.9)	0.98 ± 0.09	1.47
Diet contamination	493.4 (388.1-615.6)	1.36 ± 0.16	1.83

*Slope ± standard error.

3.5 Discussion

As shown in Chapter 2, erioglaucine was found to increase the contrast of NPV OBs when viewed under the light microscope. The images of the OBs on the membrane slides as well as on the cut out in the cap of a 0.5 ml PCR tube, shown in Chapter 2, section 2.4.2 (page 25-26), indicated that the membrane turned blue due to the erioglaucine dye. Post-LCM imaging of the cap indicated the presence of blue zones in the middle of the diet, where the size of the zone increased with an increase in the number of excisions. The increase in size of the blue zones could have occurred due to diffusion of the erioglaucine dye or "scatter" catapulting of the excisions. As such, the appearance of the blue zones on the surface of the artificial diet is a result of the accumulation of the erioglaucine from the membrane cut outs. These findings show that the OBs were successfully catapulted onto the surface of the artificial diet in the caps of the 0.5 ml PCR tubes and that the OBs are concentrated in the middle of the diet.

The frequency of lethal infection upon exposure to single LCM-captured OBs was found to be 4.83%. According to the independent action hypothesis, each virus particle has an equal, low probability of causing infection with the probability of infection being described by the Poisson distribution model (Zwart *et al.*, 2009). Therefore infection with the occlusion derived virions (ODVs) from a single OB is considered to be a rare event, which would be associated with a low percentage mortality (Zwart *et al.*, 2009). As such, previous end-point dilution studies are based on the expectation that ingestion of a single OB will result in 5% mortality (LD_5) (Arrizubieta *et al.*, 2015; Clavijo *et al.*, 2010). While this percentage of mortality is similar to that obtained using single LCM-isolated HearNPV OBs, studies conducted by Clavijo *et al.* (2010) reported a LD_5 which is significantly lower than 1, suggesting that a fraction of an OB initiated lethal infection. Since this is not possible, the findings of such studies suggest that clumps of OBs were ingested resulting in an inflation of the LD_5. The use of scanning electron microscopy (SEM) for confirmation that the OBs did not form clumps (Clavijo *et al.*, 2010) is not suitable as the OBs in suspension behave differently to that viewed

using SEM as this technique often requires pre-treatment of the sample. Furthermore, end-point dilution studies are associated with a risk that a proportion of the larvae will be exposed to no OBs or more than 1 OB based on the statistical nature of the Poisson distribution (Arrizubieta *et al*., 2015; Clavijo *et al*., 2010).

The dose-mortality response of the LCM method was compared to that of traditional bioassays. The Chi-square (χ^2) value obtained for the LCM method was found to be higher than that of the droplet feeding and diet contamination bioassays, suggesting that the data generated by the droplet feeding and diet contamination method provide a better fit of the probit model. This may have been due to the use of a smaller sample size for the LCM method as counting and isolating groups of OBs, especially for higher doses, was a lengthy process. According to Ahmed (2006), it was estimated that the isolation of 1-20 cells required a preparation time of more than three minutes. Statistical analysis of the probit-log dose regression lines revealed that the slopes were not significantly different for the various bioassay methods. As such, assays using LCM-isolated OBs produce a typical dose-mortality response indicating that the infectious properties of the OBs are uncompromised during the isolation procedure. Based on these findings, the LD_{50} value estimated for the LCM method is comparable to that estimated using traditional bioassays.

Diet contamination infection is theoretically equivalent to infection using LCM OBs as both methods were carried out using artificial diet in the caps of the 0.5 ml PCR tubes. The use of the tube lids reduces the area available for larvae to feed from, therefore the larvae used in this study were forced to feed from the top down. The LD_{50} value estimated from the diet contamination bioassay was much higher than the values from the LCM and droplet feeding bioassays. The exact location of the OBs used in diet contamination assays is unknown as the OB suspension may soak into the diet. In such assays the larvae may not always consume the entire inoculum, resulting in low precision of the administered dose (Hughes and Wood, 1981; Klein, 1978). Thus, diet contamination may provide an inflated estimate of the LD_{50} as the OBs are not ingested at the same time. Therefore, it may appear as

though more OBs would be required to initiate lethal infection in 50% of the population.

Analysis of the diet within the caps of the 0.5 ml PCR tubes indicated that the LCM excisions were confined to the middle of the diet. Since the OBs are no longer in solution, the possibility of them soaking into the diet is reduced thereby enabling synchronous intake of these particles. As such, the LD_{50} obtained using the LCM method may be expected to be closer to that of droplet feeding. However, the LD_{50} estimate of droplet feeding was found to be lower than the LCM method.

The majority of current single OB and LD_{50} studies are based on the use of droplet feeding and diet contamination. The dose administered is attained by diluting the virus stock, which is enumerated by haemocytometer counts. All dose calculations are based on the assumption that the OBs in solution follow a Poisson distribution and therefore do not form clumps and are ingested as single entities (Roberts and Coote, 1965). While OB samples are typically vortexed or subjected to sonification to avoid clumping (Biji *et al*., 2006; Kolodny-Hirsch *et al*., 1993), a fraction of the OBs in suspension may not dissociate, which not only affects enumeration but also results in clumps of OBs being ingested. Therefore, the precision of diet contamination and droplet feeding assays is restricted as these methods are subject to dose errors (Huber and Hughes, 1984). Since LCM enables inoculation with exact numbers of OBs, the technique theoretically provides the best possible representation of the frequency of lethal infection with a single OB and the LD_{50}.

In conclusion, NPV OBs isolated using LCM remain infectious post-isolation. The LCM method of inoculation can be used for empirical assessment of lethal dose values. The results of this study provide the first report of lethal infection, which is guaranteed to have occurred as a result of the ingestion of a single OB.

CHAPTER FOUR

Assessment of the suitability of DNA extracted from single, laser captured nucleopolyhedrovirus occlusion bodies for downstream amplification

4.1 Abstract

Current single occlusion body (OB) diversity studies rely on detection of variation post-passage, where lethal infection is based on the presumption that a single OB was ingested. To determine the level of diversity within a single OB, extraction of high quality DNA, which is suitable for downstream amplification, is crucial. This study aimed to assess the suitability of DNA extracted from single nucleopolyhedrovirus (NPV) OBs, isolated using laser capture microdissection (LCM), for downstream amplification. This was done by performing PCR amplification of DNA extracted from 1, 10 or 100 LCM-captured Helicoverpa armigera nucleopolyhedrovirus (HearNPV) OBs. To test whether DNA extracted from LCM-isolated OBs were suitable for amplification, a core baculovirus gene, *me53*, was amplified using PCR. Resolution of the PCR amplicons revealed that *me53* was successfully amplified in two of the eight single OB samples, and all of the samples where 10 or 100 OBs were isolated. The results of this study indicate that more reliable amplification was achieved when DNA was extracted from 10 or more LCM-isolated OBs, suggesting that the method did not inhibit DNA extraction or amplification and that amplification failure in some of the single OB samples was a result of low concentrations of template DNA. Successful DNA amplification from a single LCM-isolated OB indicates that the technique could be used to facilitate single OB diversity studies, including detection of single OB variation pre- and post-passage.

4.2 Introduction

NPVs are a group of arthropod-specific viruses, which have proven to be highly efficient biocontrol agents (Moscardi, 1999). These viruses undergo a biphasic infection cycle, which is initiated by two morphologically distinct virion types (Ikeda *et al*., 2015). Primary infection begins upon ingestion of OBs, which contain occlusion derived virions (ODVs) (Slack and Arif, 2006). The ODVs establish infection in the midgut, resulting in the production of budded virions (BVs) (Harrison and Hoover, 2012). The specialised BV phenotype enables entry into the hemocoel where systemic infection is carried out (Blissard and Theilmann, 2018).

NPV OBs are further divided into single and multiple subphenotypes (SNPV and MNPV) as they vary in arrangement of nucleocapsids (Slack and Arif, 2006). SNPVs contain a single nucleocapsid per envelope while MNPVs typically comprise 5-15 nucleocapsids per envelope (Rohrmann, 2014). This characteristic of NPV OBs enables transmission of multiple virions resulting in an increase in the multiplicity of infection (MOI) (Sanjuán, 2017). The fitness of a NPV is determined by the variants present within an OB (Wargo and Kurath, 2012). As such, the level of genetic variation that occurs within a single OB remains a key area of interest in baculovirology.

Current diversity studies are largely dependent on detection of variation post passage when lethal infection is initiated by low viral doses. Research conducted by Smith and Crook (1988), using low mortality dose infections revealed that a low frequency genotype emerged as the predominant variant after a single passage *in vivo*. Thus, it was suggested that NPV OBs contain numerous copies of a single variant. In contrast, single OB infections carried out by Clavijo *et al*. (2010) resulted in the presence of several genotypes post passage, indicating co-occlusion of multiple variants. However, the results of such studies are limited as they do not account for mutation during replication. Furthermore, the methods of infection used are based on end-point dilution, which assumes that the OBs in solution follow a

Poisson distribution (Roberts and Coote, 1965). Therefore, these studies are reliant on the probability that a larva has ingested a single OB.

Baculovirus diversity studies have been done using a range of techniques, including restriction fragment length polymorphism (Cory *et al*., 2005), sequencing (Khan *et al*., 2003) and denaturing gradient gel electrophoresis (Baillie and Bouwer, 2011), which generally detect genetic variation in sequences amplified using polymerase chain reaction (PCR) or whole genome amplification (WGA). Extraction of high quality DNA, which is suitable for downstream molecular analysis, would enable detection of variation within a single OB pre- and post-passage. While PCR has shown the ability to amplify low quantities of baculovirus DNA (Kukan, 1999, 1996), the method is prone to inhibitory substances which may originate from the sample or through introduction during processing or DNA extraction (Schrader *et al*., 2012). Such inhibitors include several organic and inorganic compounds, which prevent amplification by direct interaction with the template DNA or by obstructing a step in the PCR reaction (Moreira, 1998). This may result in partial amplification, due to decreased sensitivity, or complete inhibition giving rise to false negative results (Schrader *et al*., 2012).

In Chapter 2, a LCM method incorporating the use of erioglaucine, was developed for the isolation of NPV OBs from solutions. The use of an ultraviolet (UV) laser-based system, such as that used in the developed method, enables isolation of microscopically visualised particles which are loaded onto polymer membranes, typically composed of polyethylene naphthalate (PEN) or polyethylene tetraphthalate (PET) (Vogel *et al*., 2007). Microdissection is carried out by a phenomenon known as "cold ablation", which ensures that the sample as well as adjacent matter remain unaffected as the time available for heat transfer is < 1 µs (Burgemeister, 2005; Vogel *et al*., 2007). While the infectious properties of LCM-captured OBs are preserved post-isolation (described in Chapter 3), it is not known whether the erioglaucine or the PEN membrane used to isolate single OBs has inhibitory effects on DNA extraction or downstream amplification. This study

aimed to assess the suitability of DNA extracted from single LCM-isolated NPV OBs for downstream amplification, using a PCR approach.

4.3 Materials and Methods

4.3.1 Insect rearing

H. armigera eggs from the Agricultural Research Council (Pretoria, South Africa) were used to set up permanent laboratory cultures. The cultures were maintained as described in Chapter 2, section 2.3.1 (page 20).

4.3.2 Virus propagation and purification

Third instar (± 6 d-old) *H. armigera* larvae were used to propagate HearNPV (a SNPV) as described in Chapter 2, section 2.3.2 (page 20). Isolation of OBs from suspensions was carried out using the gradient-purified sample.

4.3.3 LCM

HearNPV OBs, viewed using the 100× objective, were isolated using the PALM MicroBeam IV laser-capture microdissection system as described in Chapter 2, section 2.3.4 (page 21). Initial findings indicated that DNA extraction and PCR could not be carried out using OBs catapulted directly into the caps of 0.5 ml PCR tubes (data not shown). The OBs were therefore catapulted into the caps of 0.5 ml PCR tubes, which contained 57 µl of sdH_2O. Eight replicates of single OBs and triplicates which contained 10 and 100 OBs were collected. Six replicates of control cut outs were also obtained.

4.3.4 DNA extraction

Genomic DNA was extracted from the LCM OBs using the NucleoSpin® Tissue XS kit (Macherey- Nagel), based on the instructions described in the user manual for extraction of genomic DNA from tissue. This kit was chosen as it enables DNA extraction from small sample quantities (Macherey- Nagel). Tubes containing LCM OBs were subjected to centrifugation at 10 000 ×*g* for 30 s, to ensure that the

suspension containing the OBs was present at bottom of the tube. OBs were subjected to an initial pre-treatment. This included the use of sodium carbonate (added to make up a final concentration of 50 mM), which enabled dissolution of the polyhedron component of the OBs. The suspension was incubated at 37 °C for 30 min, after which 20 µl of 1 M Tris-HCl (pH 7) was added. The suspension was treated with 8 µl of a 10% (w/v) SDS solution and incubated at 37 °C for 30 min, to facilitate membrane disruption of the ODVs. The DNA was then extracted from the suspension based on the instructions described in the user manual. The DNA was eluted from the column using 20 µl of elution buffer. The DNA samples were stored at -20 °C.

4.3.5 PCR

To test whether DNA extracted from single LCM-isolated OBs is suitable for amplification, a core baculovirus gene, *me53* was amplified using PCR. A 349 bp region of the *me53* gene (1080 bp) was amplified using *me53* specific primers (MS1-F: 5'-CAGCGTCGCTAGTTAACC-3'; MS1-R: 5'-TGGATGTAATATGTA AGTTTGAGC-3') (D. Kitchin, unpublished primers). IDT software (Coralville, USA) was used to identify essential oligonucleotide properties such as melting temperature and structural mismatches. In addition, BLAST analysis was used to validate the specificity of the primer sets for the reference HearNPV genome (GenBank accession number: NC_003094.2). PCR amplification was conducted with the aid of the FastStart Taq DNA Polymerase kit (Roche). This enzyme was chosen due to its high stability at room temperature, as it remains inactive at temperatures below +75 ºC. Amplification reactions were set up according to the manufacturer's instructions, with the addition of 5 µl of template DNA to each 25 µl reaction volume. The PCR programme was as follows: initial denaturation at 95 °C for 5 min; 35 cycles each consisting of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s; a final extension step at 72 °C for 5 min. A positive control consisting of DNA isolated from OBs in suspension and a negative PCR control, containing 5 µl sdH_2O instead of DNA as a template, was included. The sizes of the amplicons were evaluated by agarose gel electrophoresis

using 1% (w/v) agarose gels stained with GelRed® (Biotium), with a run time of 45 min at 90 V.

4.4 Results

PCR amplification of all DNA samples and controls was conducted on the same day. However, due to the limitation in the number of lanes in a single gel, the products were evaluated using two separate gels (Figure 5 and 6), where the amplicons from all of the single OB samples and representatives of the amplicons from 10 and 100 OBs as well as the negative controls were shown. The *me53* primer sets produced bands of approximately 350 bp, which matched the expected size thereby indicating successful amplification. DNA extracted from single OBs produced faint bands in two of the samples (Figure 5 and 6). This matched the intensity of the bands produced from 10 OBs but was lower than the intensity of the bands produced from 100 OBs (Figure 5 and 6).

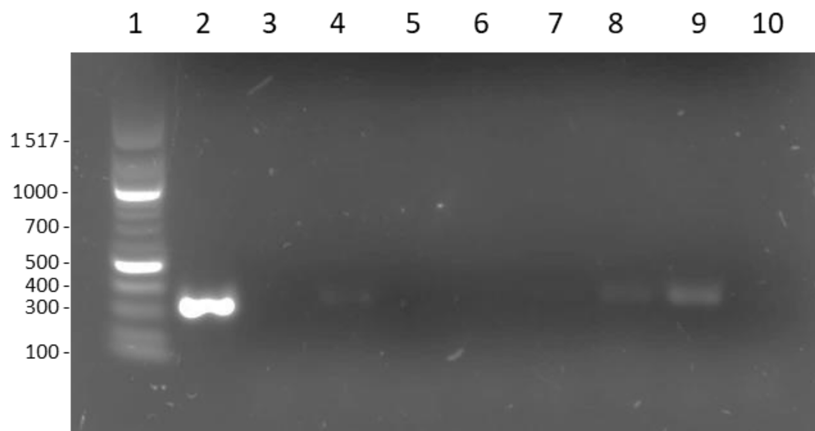

Figure 5. Agarose gel (1% w/v) electrophoresis of set 1 of PCR amplicons. Lane 1: molecular weight marker (size in bps); Lane 2: positive control, where template DNA with a concentration of 8.4 ng/µl was used; Lane 3: no template control; Lanes 4-7: single LCM OB; Lane 8: 10 LCM OBs; Lane 9: 100 LCM OBs; Lane 10: LCM negative control, where the template used was obtained from control (no OB) cut outs which were subjected to the DNA extraction protocol.

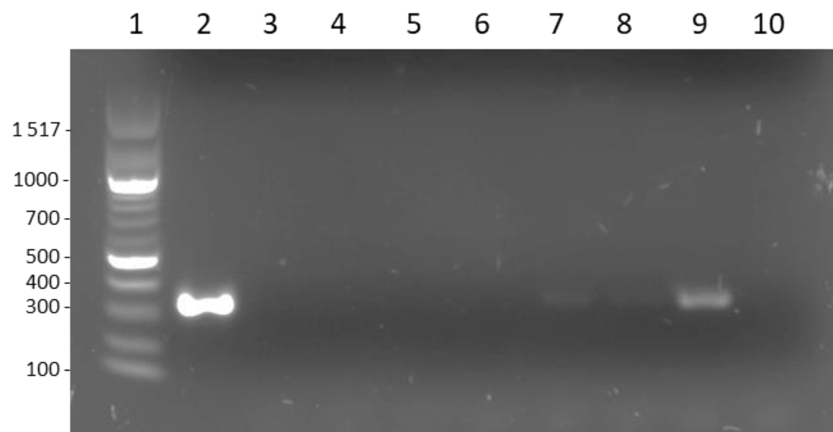

Figure 6. Agarose gel (1% w/v) electrophoresis of set 2 of PCR amplicons. Lane 1: molecular weight marker (size in bps); Lane 2: positive control, where template DNA with a concentration of 8.4 ng/µl was used; Lane 3: no template control; Lanes 4-7: single LCM OB; Lane 8: 10 LCM OBs; Lane 9: 100 LCM OBs; Lane 10: LCM negative control, where the template used was obtained from control (no OB) cut outs which were subjected to the DNA extraction protocol.

4.5 Discussion

The *me53* PCR amplicons, from the LCM-isolated OBs, produced bands of approximately 350 bp, which was around the size expected (349 bp). The band was evident in two of the eight samples where a single OB had been isolated, and all three of the samples where 10 or 100 OBs were isolated. As shown by the examples of the LCM negative controls, bands were not produced indicating that amplification did not occur when extracts from control cut outs were used as a template. While amplification occurred in all of the samples where DNA was extracted from 10 or 100 OBs, this was not apparent in the single OB samples, suggesting that amplification failure was a result of low concentration or lack of template DNA in some of the single OB samples. This could have occurred due to a loss of template DNA during the pre-treatment or absence of the OB in the droplet of water (possibly due to the OB sticking to the surface of the tube).

The intensity of the bands formed for all of the samples was much lower than that of the positive control, suggesting that the concentration of the template DNA was much lower than 8.4 ng/µl. The Qubit method of DNA quantitation is considered to be the benchmark method due to its ability to distinguish between nucleic acids, free nucleotides and contaminants (O'Neill *et al*., 2011). Qubit analysis of the DNA samples from the LCM OBs indicated that the concentration of the DNA was below the 0.50 ng/ml detection limit of the apparatus (data not shown). Therefore, the amount of DNA extracted from the LCM-isolated OBs could not be determined.

PCR is considered to be the gold standard method of detection as it has shown the ability to amplify picogram to femtogram quantities of baculovirus DNA (Burden *et al*., 2002; Kukan, 1999, 1996). While the use of the technique has been associated with a wide range of detection limits for various baculovirus isolates, a study conducted by Burden *et al*. (2003) found that the detection limit was as low as 3.8 fg (~ 20 genome copies) for Mamestra brassicae multiple nucleopolyhedrovirus. HearNPV OBs, such as that isolated in this study, contain approximately 31 ODVs per OB (Sun *et al*., 2004), therefore at most 31 copies of genomic DNA were

extracted and amplified in some of the single LCM-isolated OB samples. Amplification failure in the remaining single OB samples could have occurred as the concentration of DNA extracted in these replicates was below the amplification limit. To increase the sensitivity of detection, a quantitative PCR (qPCR) approach, with a detection limit as low as 5 viral genomes (Graham *et al*., 2015) could be used as this would enable monitoring of amplification and subsequent quantification of the products in real time (Bustin, 2010).

An increase, compared to the single OBs, in the number of OBs isolated led to an increase in the intensity of the bands formed. Assuming 100% DNA extraction from the LCM-isolated OBs, a 10-fold increase in the number of OBs resulted in a 10-fold increase in the amount of template DNA for subsequent amplification. As such, approximately 310 copies were isolated and amplified from 10 OBs and 3100 copies from 100 OBs. Due to the exponential nature of PCR (Kramer and Coen, 2001), the presence of more template DNA results in the production of more amplicons. While an increase in the number of amplicons could not be seen by a difference in the intensity of the bands produced in the single LCM OB and 10 LCM OB samples, it was evident as amplification occurred in all of the 10 LCM OB samples. Therefore, PCR was more reliable as the number of OBs increased as more copies of DNA were successfully extracted and amplified in all of the replicates where 10 or 100 OBs were captured.

The results of this study suggest that the erioglaucine and PEN membrane used to isolate OBs from suspension had no inhibitory effects on DNA extraction or downstream amplification and that amplification failure was a result of low template concentrations. These findings indicate that LCM could be used to isolate single OBs for subsequent amplification, which would enable detection of genetic variation. The use of the LCM method not only overcomes the limitations associated with end-point dilution (Huber and Hughes, 1984), but also creates the possibility of detection of genetic variation in single OBs pre- and post-passage.

In conclusion, DNA extracted from single LCM-isolated OBs is suitable for downstream amplification. The technique used in this study provides the framework for future single OB diversity studies.

CHAPTER FIVE

General discussion and conclusion

Dose-response relationships are crucial to understanding the virulence of nucleopolyhedroviruses (NPVs) as such relationships provide an indication of the link between exposure to a certain pathogen and the probability that lethal infection will occur (Pujol *et al*., 2009). To provide the best representation of the minimum number of occlusion bodies (OBs) required to initiate lethal infection, a precise method of inoculation is required. Current low dose studies are based on the use of droplet feeding, diet surface contamination or diet incorporation bioassays (Hughes and Wood, 1981; Ignoffo, 1965). While the use of these methods suggest that the number of OBs required to initiate lethal infection is low (Baillie and Bouwer, 2013; Clavijo *et al*., 2010; Figueiredo *et al*., 1999), these methods rely on the use of end-point dilution, which is of statistical nature (Gross *et al*., 2015). As such the precision of the techniques is limited as the they are subject to dose errors (Huber and Hughes, 1984). This suggests that the development of a highly sensitive OB isolation technique, which enables empirical assessment of the lethal dose would provide a significant contribution to understanding the virulence of NPVs.

A laser capture microdissection (LCM) technique was developed to isolate single NPV OBs from suspensions (Chapter 2). It was found that the polyethylene naphthalate (PEN) membrane slides on which the sample was spotted onto inherently contains circular pores with a similar size range to the OBs. A common dye used to assess larval feeding, erioglaucine, was found to increase the contrast of the OBs on the membrane, making the particles more distinguishable. Single OBs, characterised by a dark solid outline, were laser catapulted into the caps of 0.5 ml PCR tubes. Post-microdissection analysis of the caps revealed the presence of membrane cut outs containing single OBs. This indicated that a laser microcapture method proved to be suitable for the isolation of single OBs from suspensions.
The *in vivo* infectious properties of the NPV OBs isolated, using the developed LCM method, was assessed in Chapter 3. Exposure of second instar *Helicoverpa*

armigera larvae to single LCM-isolated OBs resulted in a frequency of lethal infection of 4.83%. To validate the use of the technique in dose-response assays, the slope of the probit-log dose regression line from the LCM method was compared to that of traditional bioassays namely, droplet feeding and diet contamination. It was found that the slopes of the probit-log dose regression lines for the different bioassay methods were not significantly different, indicating that inoculation using the LCM method produced a typical dose-mortality response. The LD_{50} estimate obtained for the LCM method was found to be 188.7 OBs, which was higher than the 89.6 OBs obtained for droplet feeding but lower than the 493.4 OBs obtained for diet contamination. While diet contamination and droplet feeding assays are of statistical nature (Ridout and Fenlon, 1991; Roberts and Coote, 1965), the LCM method enables inoculation with exact numbers of OBs and therefore theoretically provides the best representation of the frequency of lethal infection with a single OB and the LD_{50}. Based on these findings, the infectious properties of the OBs remain unaffected by the LCM isolation procedure.

To determine whether DNA extracted from single LCM-isolated OBs was suitable for amplification, a core baculovirus gene, *me5*, was amplified using PCR (Chapter 4). PCR amplification was carried out using DNA extracted from 1, 10 or 100 OBs. Amplicons were detected in two of the eight single OB samples and all of the samples where DNA was extracted from 10 or 100 LCM-isolated OBs. While amplification failure occurred in some of the single OB samples, more reliable amplification was achieved when DNA was extracted from 10 or more LCM-isolated OBs. These findings suggest that LCM isolation did not inhibit downstream amplification and that amplification failure in some of the single OB samples may have been a result of the number of copies of template DNA extracted being below the amplification limit of the standard PCR approach used. Successful amplification of DNA extracted from a single LCM-isolated OB indicates that the use of the technique is not restricted to infection studies but can also be used to facilitate molecular analysis studies.

Future studies could be done using LCM to isolate OBs for assessment of genetic variation within a single OB both pre- and post-passage. This could be done using PCR amplification of core baculovirus genes, such as *DNA polymerase* or *me53* (Baillie and Bouwer, 2012b), where genetic variation in the genes could be detected using denaturing gradient gel electrophoresis (DGGE). The LCM technique could be used in conjunction with whole genome amplification and sequencing approaches to assess the overall genetic variation within a single OB. The concentration of DNA extracted from a single OB could be determined by qPCR.

A LCM method was successfully developed for the isolation of single infectious NPV OBs from suspensions. The method will facilitate future studies, such as those which incorporate the use of PCR and DGGE, to assess the genetic diversity of the occlusion derived virions in an OB. Overall, the method developed in this study has the potential to make a significant contribution to the field of baculovirology.

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