The Immunogenicity of Plant-produced Human Papillomavirus (HPV) Virus-like particles (VLPs) in Mice

By

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In memory of Linda Nduuviteko Uuyage and Konias Amakali Athingo

Declaration

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Table of Contents

Abstract

Cervical cancer is caused by infection with high-risk Human papillomaviruses (HPVs). It is ranked fourth among the top cancers in women worldwide, with ~87% of the global cervical cancer cases reported in developing countries. The HPV L1 capsid protein can self-assemble into virus-like particles (VLPs) that are structurally like native virions, which is the foundation on which commercially available vaccines have been developed. There are 3 commercially available HPV vaccines that are effective at preventing HPV infections, but are expensive, therefore limiting their use in the poorer developing countries where they are most needed. Thus, there is a need for more cost-effective HPV vaccines for use in these countries. Over the years, the use of plants to produce vaccines has begun to be more favourably looked upon as a cost-effective alternative to conventionally used expression systems. The aim of this study was to evaluate the plant-based transient expression system as a tool to produce potentially cost-effective HPV L1 VLP-based vaccines, particularly for developing countries.

Firstly, the L1 proteins of the 8 most common high-risk Human papillomavirus types in Africa (HPV 16, 18, 31, 33, 35, 45, 52, and 58) and 2 low risk types (HPV 6 and 34) were transiently expressed in *Nicotiana benthamiana*. The proteins were purified via isopycnic ultracentrifugation using sucrose and Optiprep™ density gradients, and the assembly of VLPs assessed by transmission electron microscopy (TEM). To further assess whether the VLPs are immunogenic, HPV 35, 52 and 58 were selected for mice studies. These were selected in particular, as HPV 35 is the fifth most prevalent type in Africa, and HPV 52 and 58 are among the most frequently reported high-risk types in Sub-Saharan Africa. VLPs representing the 3 HPV types were quantified and prepared for immunization in mice. The commercially available Gardasil® HPV VLP vaccine was used as a positive control. The immunogenicity of the vaccines was evaluated by testing for the presence of anti-L1 antibodies in sera from immunized mice using enzyme-linked immunosorbent assays (ELISAs) and western blots. Sera from immunized mice were also tested for the presence of neutralizing antibodies using pseudovirion based neutralization assays (PBNAs).

L1 proteins of all 10 HPV types tested were successfully expressed in *N. bethamiana,* and TEM analysis showed that expression resulted in the successful formation of fully

assembled VLPs (40-60nm) as well as small VLPs and/or capsomeres (25-39nm). The analysis of the immune response showed that type-specific L1-specific antibodies were produced which were able to successfully neutralize homologous pseudovirions (PsVs) in PBNAs. Sera from mice immunized with plant-produced VLPs were further tested against heterologous HPV 6, 16, 18, 31, and 45 PsVs. However, none of the tested heterologous HPVs were neutralized, suggesting that plant-made VLPs induced type-specific neutralizing antibodies only.

In conclusion, this study successfully demonstrated the potential for using plant-based transient expression systems to produce affordable and immunogenic HPV vaccines, particularly for developing countries. This is the first study describing the expression of 10 HPV L1 proteins in plants, marking a step towards the development of cheaper HPV vaccines which could be combined to generate an effective multivalent vaccine against HPVs.

Abbreviations

AAV	Adeno-associated virus
AEC	Animal research ethics committee
ANOVA	Analysis of variance
BCIP/NBT	5-bromo-4-chloro-3-indoxyl-phosphate nitroblue tetrazolium
bp	Base pairs
BRU	Biopharming research unit
BSA	Bovine serum albumin
BSL	Biosafety level
CD	Cluster of differentiation
CIN	Cervical intraepithelial neoplasia
COPV	Canine oral papillomavirus
CP	Coat protein
CPMV	Cowpea mosaic virus
CRPV	Cottontail rabbit papillomavirus
CTB	Cholera toxin B
CysPs	Cysteine proteinases
DC	Dendritic cell (s)
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco’s phosphate buffered saline
dpi	Days post infiltration
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzymes-linked immunosorbent assay (s)
EV	Empty vector
FDA	Food and Drug Administration
g	Grams
GCD	Glucocerebrosidase
HEK	Human embryonic kidney
HPV	Human papillomavirus (s)
Hsp	Heat shock protein
HSPBS	High salt phosphate buffered saline
HSPG	Heparan sulfate proteoglycans
HSV	Herpes simplex virus
Kb	kilobase (s)
kDa	Kilodalton (s)
kg	Kilogram (s)
kPa	kilopascal
kV	Kilovolt (s)
Lab	Laboratory
LB	Luria-Bertani
LSD	Lysosomal storage disorder
M	Molar (s)
MAb	Monoclonal antibody
MES	2-morpholineethanesulforic acid
mg	Milligram (s)
$MgCl_2$	Magnesium chloride

MHC	Histocompatibility complex
ml	Millilitre (s)
mM	Millimolar (s)
NAb	Neutralizing antibody
NaCl	Sodium chloride
ND	Nuclear domain
ng	Nanograms
nm	Nanometre (s)
OD	Optical density
ORF	Open-reading frame (s)
PAGE	Polyacrylamide gel electrophoresis
PBNA	Pseudovirion-based neutralization assay (s)
PBS	Phosphate buffered saline
PC	Proprotein convertase
PCR	Polymerase chain reaction
pH	Potential hydrogen
PS	Plant serum
PsV	Pseudovirion (s)
PVX	Potato X virus
RLU	Relative light unit
RNA	Ribonucleic acid
ROPV	Rabbit oral papillomavirus
SAB	Sample application buffer
ScFv	Single chain variable fragment (s)

SDS	Sodium dodecyl sulfate
SEAP	Secreted alkaline phosphatase
SV40	Simian virus 40
TA	Tissue antigen
T-DNA	Transfer DNA
TEM	Transmission electron microscopy
Ti	Tumor inducing
Tk	Thymidine kines
TLR	Toll-like receptor
TMV	Tobacco mosaic virus
tRNA	Transfer RNA
UCT	University of Cape Town
URR	Upper regulatory region
USA	United States of America
UTR	Untranslated region
v/v	Volume per volume
vir	Virulence
VLP	Virus-like particles (s)
w/v	weight per volume

Symbols

α	Alpha
~	Approximately
°C	Degrees Celsius
µF	Microfarad

µg	Microgram (s)
µl	Microlitre (s)
µM	Micromolar (s)
Ω	Ohm (s)
%	Percentage (s)

1. Chapter 1: Literature Review

1.1 Introduction

Cervical cancer is ranked fourth among the top cancers in women and is the main cause of mortalities due to cancer among women of developing countries (Shrestha *et al.*, 2018, Ferlay *et al.*, 2015). In low- and middle-income regions, cervical cancer is ranked the second most common cancer with ~570 000 new cases reported in 2018, which attribute to 84% of worldwide cervical cancer cases. Approximately 311 000 cervical cancer deaths were reported in 2018, with more than 85% deaths occurring in developing countries where screening and vaccination programmes are insufficient. This represents about 7.5% of all cancer deaths in females. In 2018, 62 170 prevalent cases (16.1% worldwide prevalence) were reported in Africa, with 8 593 cases reported in South Africa (Ferlay *et al.*, 2018).

Cervical cancer is caused by infection with high-risk HPVs (Ladd *et al.*, 2019, Zur Hausen, 1996). Ninety nine percent of biopsy specimens from cervical cancers have been proven to contain HPVs, with HPV 16 being the most frequent, followed by HPV 18 (Li *et al.*, 2011). HPV 16 and HPV 18 are estimated to account for 70% of all cervical cancers worldwide, with other high-risk types (HPV 31, 33, 35, 45, 52 and 58) accounting for an additional 20% of cervical cancer worldwide (Ferlay *et al.*, 2018).

The causal linkage of HPVs to cervical cancer have attracted scientific attention. This has led to enhanced research in this field and as a result, three efficient prophylactic vaccines have been developed. Although cervical cancer deaths have been strongly reduced by using these HPV preventative vaccines, and screening for cervical cancer and timely treatment in industrialized countries, access to such services is a big challenge in developing countries (Denny, 2006, Moodley *et al.*, 2006). Consequently, cervical cancer still remains a significant public health challenge in resource-poor countries (Franco and Harper, 2005). Therefore, there is still much to do to reduce HPV infections and cervical cancer deaths, especially in developing countries. This literature will review HPV as the main causative agent of cervical cancer, vaccines that are available to reduce cervical cancer deaths and challenges associated with these vaccines.

1.2 The genome and classification of HPV

1.2.1 The structure of HPV

HPVs are small viruses that infect epithelial cells and have a 8 kb non-enveloped double-stranded circular deoxyribonucleic acid (DNA) genome (Mahdavi and Monk, 2005, De Villiers *et al.*, 2004). The genome has eight open-reading frames (ORFs) separated into three regions: the early (E) region which encodes the virus replication proteins (E1, E2, E4-E7); the late (L) region encoding the capsid proteins, the major L1 and the minor L2 structural proteins which are crucial for virion assembly; and the upper regulatory region (URR) which contains *cis* elements and is essential for viral replication and transcription (Figure 1.1) (Stanley, 2012).

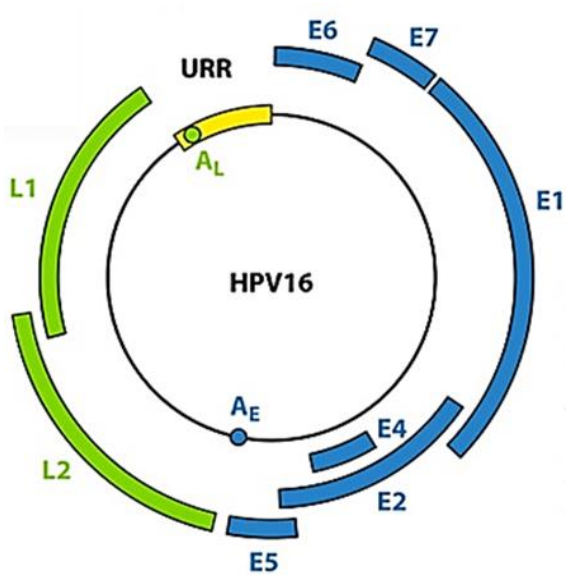


Figure 1.1| HPV genomic structure: The map of a typical high-risk HPV genome depicting the early genes E1, E2, E4-E7, the late genes encoding the L1 and L2 structural proteins and the URR. A_L is the late polyadenylation site while A_E is the early polyadenylation site. Image modified with permission from Stanley (2012).

The HPV capsid is made up of two proteins: the L1 major capsid protein and L2 minor capsid protein (Pereira *et al.*, 2009, Chen *et al.*, 2000, Kirnbauer *et al.*, 1993). The L1 structural protein is approximately 56 kDa, while the L2 minor capsid protein is estimated at 64-78 kDa (Hagensee *et al.*, 1994, Jin *et al.*, 1989, Doorbar and Gallimore, 1987). The virion, measuring 50-60nm in diameter, contains 360 molecules of L1, arranged in 72 pentamers with an average of 30 L2 molecules (Buck *et al.*,

2008, Trus *et al.*, 2005) . Interaction among the 72 pentamers of L1 result in the T=7 icosahedral symmetry of the virion (Figure 1.2) (Chen *et al.*, 2000).

T=7

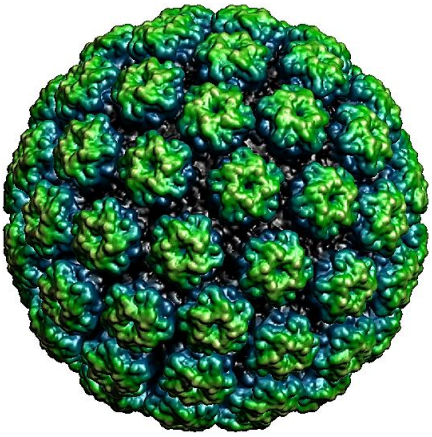


Figure 1.2| Atomic model of HPV 16 L1 capsid protein: The virion T=7 icosahedral symmetry. A 72-pentamer particle made up of full-length L1. Model obtained from http://viperdb.scripps.edu (Shepherd *et al.*, 2006).

1.2.2 Classification

Traditionally, papillomaviruses and polyomaviruses belonged to one family, the *Papovaviridae*, as these two groups similarly have non-enveloped circular double stranded DNA genomes. Nevertheless, it was later found that papillomaviruses and polyomaviruses have different genome sizes and organizations, and do not share any resemblances in amino acid sequences, therefore, they are now recognized as two distinct families: *Papillomaviridae* and *Polyomavidae,* respectively (De Villiers *et al.*, 2004).

Currently 16 genera are completely described in the *Papillomaviridae* family (Figure 1.3). Among them, five genera known as Alpha-papillomavirus, Beta-papillomavirus, Gamma-papillomavirus, Mu-papillomavirus and Nu-papillomavirus are found in humans and are denoted as HPVs (De Villiers *et al.*, 2004). The Alpha genus group contains both the high risk and low-risk mucosal types which cause cervical cancer and genital warts, respectively, whereas other genera consist of cutaneous HPV types (Doorbar *et al.*, 2012).

Scientists have been using the highly conserved gene of the papillomaviruses, the L1 ORF in the identification of new papillomavirus types (De Villiers *et al.*, 2004).

Presently, more than 118 HPV types have been identified and described according to their oncogenic potential and biological niche (Zandi *et al.*, 2010, De Villiers *et al.*, 2004). Moreover, based on the molecular and epidemiological evidence, HPV types are divided into low-risk (mostly found in genital warts and are known to cause benign lesions) and high-risk types (known to cause malignant lesions and mainly linked with invasive cervical cancer) (Muñoz *et al.*, 2003). Among the low risk HPV types are HPV 6, 34 and 11, and the high risk types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (Zandi *et al.*, 2010).

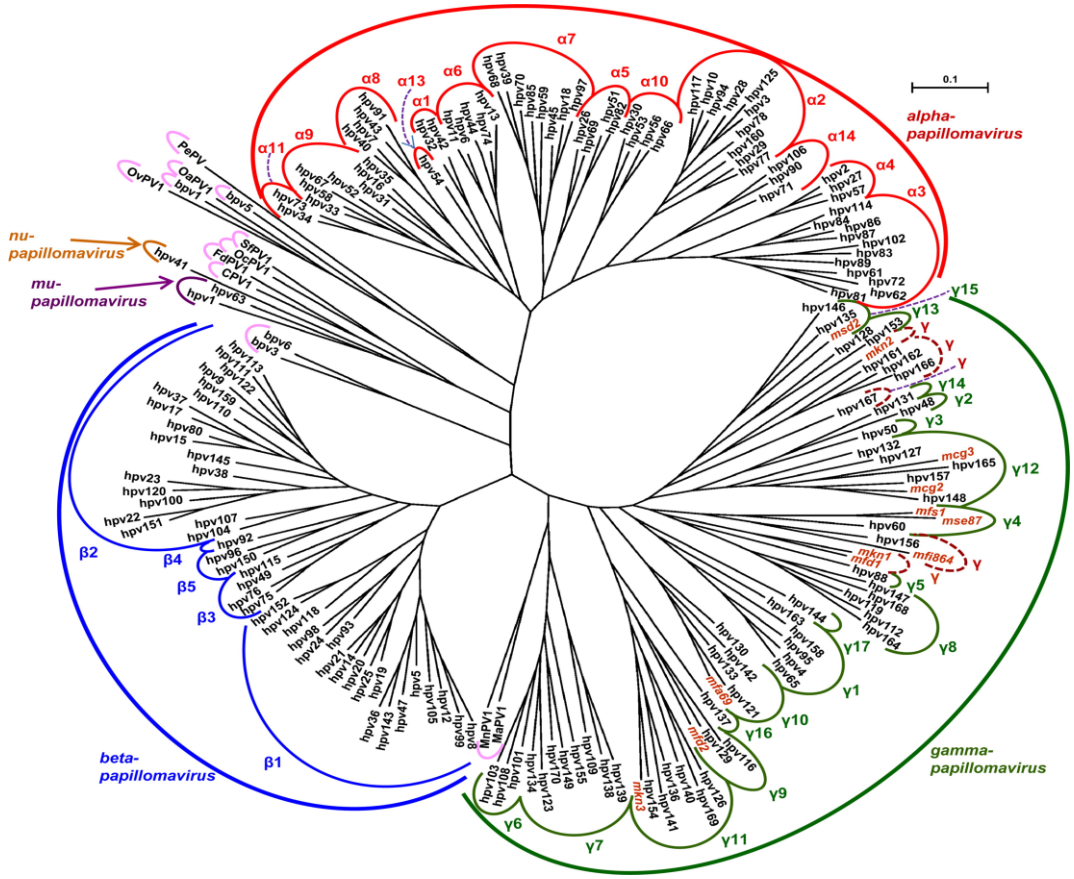


Figure 1.3| Taxonomic classification of papillomaviruses: Phylogenetic tree of genera and species of papillomavirus based on the ORF sequences of L1. Tree obtained with permission from de Villiers (2013).

1.3 HPV life cycle

1.3.1 Viral Entry

HPVs are intracellular parasites, therefore they must be able to insert their genomes into the host target cells to recruit cellular machinery for replication (Marsh and

Helenius, 2006). HPV virus particles enter the basal keratinocytes of the infected epithelium, with the viral structural proteins playing essential roles during viral infection (Roberts *et al.*, 2007). The development of organotypic raft cultures and later VLPs and PsVs have allowed researchers to produce infectious virus particles and therefore study the HPV life cycle *in vitro*.

Initial contact between the virus and the host cell during *in vivo* infection is initiated by binding of the virus to the basement membrane (Figure 1.4) (Kines *et al.*, 2009, Day *et al.*, 2008, Richards *et al.*, 2006). Figure 1.4 shows a series of steps involved during viral infection. The initial interaction between the virus and the basement membrane causes conformational changes in the virion which is essential for viral infection (Kines *et al.*, 2009). Conformational changes in the virion exposes L2 cross-neutralization epitopes to furin and/or proprotein convertase (PC) 5/6 cleavage and enables transfer of the virus to cell receptors (Kines *et al.*, 2009).

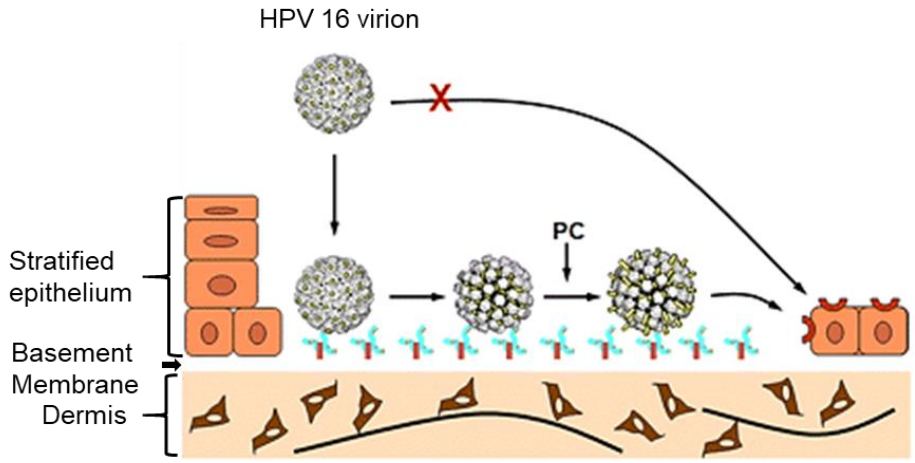


Figure 1.4| Schematic model of initial steps during natural infection: The attachment of virion on the basement membrane exposes L2 to furin cleavage and ultimately transfer to cell receptors. Image adapted with permission from Kines *et al.* (2009).

In contrast to natural infection, the initial contact between the HPV capsid and the host cell in *in vitro* is initiated by binding of the HPV capsid on the cell surface receptors (Kines *et al.*, 2009, Roberts *et al.*, 2007, Giroglou *et al.*, 2001a, Joyce *et al.*, 1999). Particularly, during *in vitro* infection , the initial interaction depends on the attachment of the major L1 capsid protein on the cell surface heparan sulfate proteoglycans (HSPG) (Kines *et al.*, 2009, Drobni *et al.*, 2003, Giroglou *et al.*, 2001b). Notably, *in*

vitro HPV can also bind to a receptor found in the extra-cellular matrix (ECM) known as laminin-5. However, the interaction with laminin-5 is not as important for efficient HPV infection as the HSPG interaction (Culp *et al.*, 2006a, Culp *et al.*, 2006b).

The L1 interaction with HSPG causes conformational changes to the viral capsid before cell entry. Specifically, the surface binding exposes the L2 N-terminus to furin cleavage (Day *et al.*, 2008, Richards *et al.*, 2006). Furin cleavage of L2 occurs at a furin consensus site which is preserved in all papillomaviruses (Richards *et al.*, 2006). This binding causes another conformational change and exposes the secondary cell receptor binding sites to the L2 molecules (Day *et al.*, 2008, Day *et al.*, 2007). The main difference between *in vivo* and *in vitro* infection is the conformational change of L2 molecule that occur on the basement membrane, and after cell surface adsorption respectively (Day *et al.*, 2008).

After HPV virions successfully attach to the cell surface receptors, they are translocated into the cell to begin the infection. Currently, the entry mechanisms associated with HPV interaction with the target cell are still a topic of scientific discussion (reviewed in Horvath *et al.* (2010)). Non-enveloped viruses are known to use either caveolae or clathrin mediated endocytic pathways to successfully infect cells (Smith and Helenius, 2004). Studies suggest that efficient cell entry of HPV virions comprises translocation by endocytosis. Studies that investigated HPV 16 entry reported the association of clathrin-dependent endocytosis (Spoden *et al.*, 2008, Bousarghin *et al.*, 2003, Day *et al.*, 2003), while caveolar-mediated mechanisms were observed in HPV 31 (Smith *et al.*, 2007b, Bousarghin *et al.*, 2003).

1.3.2 HPV as an infectious agent

HPVs are infectious agents which are categorized by their tissue tropism and species specificity (Stanley, 2006). The HPV infectious cycle entirely depends on the host keratinocyte cell replication and differentiation activities of the target cell (Stanley, 2012). This has hindered the study of the virus's infectious cycle until the development of the organotypic raft culture system. This system allowed the establishment of an *in vitro* system which mimics *in vivo* epithelial features and is able to reproduce the complete life cycle of HPV (Kreider *et al.*, 1987). This system allows HPV genomic DNA to be introduced into keratinocytes and isolate cells that are like undifferentiated basal cells. HPV cell lines used in this system are obtained either from infected

epithelial tissues or produced by introducing HPV genomic DNA into keratinocytes (McLaughlin-Drubin and Meyers, 2005).

HPV requires epithelial micro-abrasions for viral entry to the basal epithelial cells (Doorbar *et al.*, 2012, Conway and Meyers, 2009). The life cycle of HPV is separated into the non-productive phase and the productive phase (Figure 1.5) (Stanley, 2006).

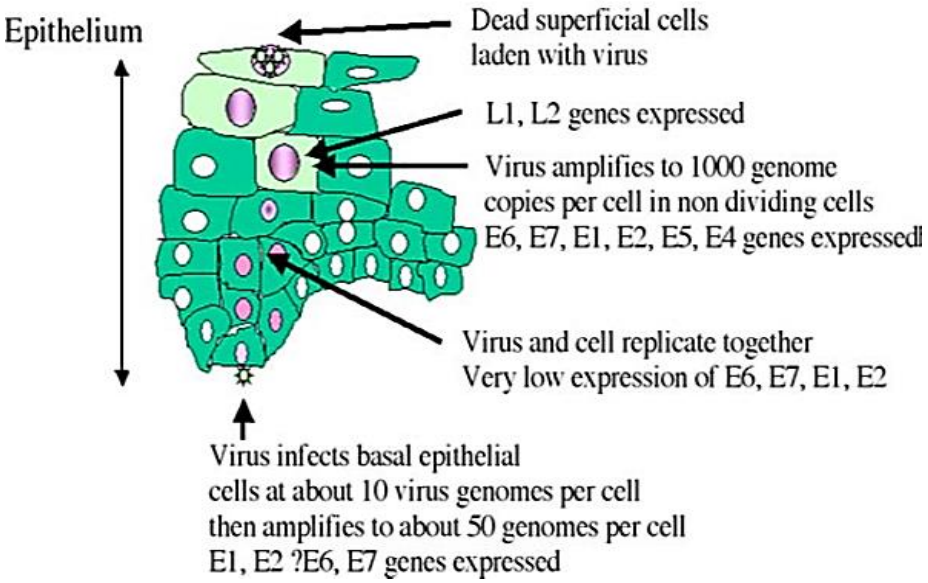


Figure 1.5| Infectious HPV life cycle: The virus gains entry to the basal cells of the epithelial via micro-abrasions. The non-productive stage of the life cycle happens in the proliferative compartment of the epithelium. Viral gene expression and DNA amplification of thousands of genome copies occurs in the productive stage of the life cycle. Late virus proteins such as L1, L2, and E4 are synthesized, and eventually virions are assembled. Image taken with permission from Stanley (2006).

The non-productive phase, also known as the phase of plasmid maintenance, occurs immediately after the virus enters the basal layer of epithelial cells. The virus synthesizes its DNA once per cycle of host replication, hence the genome is sustained as a low copy number episome (Doorbar *et al.*, 2012). The early E1 and E2 proteins of the virus are expressed during this phase. These proteins are necessary for viral DNA replication, because together they form a complex that has the origin of replication and recruit the host cell replication machinery (Conway and Meyers, 2009). During this phase, the virus gene expression is highly controlled, thus the E6 and E7 oncogenes are poorly expressed (Stanley, 2012).

The viral productive phase happens in the surprabasal layers of epithelial cells. Here the virus amplifies its DNA using the host replication tools to yield many viral genomes. Late proteins, such as L1, L2 and E4, are expressed in this phase and eventually lead to viral packaging and assembly (Stanley, 2006, Heino *et al.*, 2000). In this phase all viral genes are expressed and leave the cell as infectious viral particles (Stanley, 2012). Keratin cells collapse after the E1-E4 proteins interconnect their networks, therefore, letting mature virions escape (Conway and Meyers, 2009). It takes approximately 3 weeks for these infectious virus particles to be synthesized (Stanley, 2012).

Normally, after cell division of basal cells, daughter cells disconnect from the basement membrane, and move to the suprabasal compartments to initiate differentiation (Fehrmann and Laimins, 2003). In contrast, HPV infected suprabasal cells do not leave the cell cycle, but remain to support HPV DNA synthesis and eventually HPV virions (Doorbar *et al.*, 1997).

1.3.3 Virion assembly and maturation

An effective virus is one that is able to produce new viral components (transcription and translation) inside the host cell and assemble them to mature into stable virions which can effectively infect other hosts. Most viruses use chaperones or scaffolding proteins to successfully accomplish the task of assembly (Villarreal, 2004).

Failure to grow efficient amount of the virus in cultures has greatly affected the studies of capsid assembly of the HPV virion. This is because viral DNA replication and transcription are tightly dependent on the host target cell differentiation activities (Chen *et al.*, 2000). Molecular interactions required for HPV assembly are not well understood (Conway and Meyers, 2009). Furthermore, the role of the minor L2 structural protein in HPV assembly is not well understood (Buck *et al.*, 2005b).

It is reported in literature that the viral genome can be packaged into VLPs made of both the L1 and L2 proteins, however not into L1 composed capsids (Heino *et al.*, 2000). This partly suggests that the L2 minor capsid protein is essential for HPV virion assembly, specifically the viral genome encapsidation (Holmgren *et al.*, 2005, Heino *et al.*, 2000). Although it is still unknown how this capsid protein facilitates encapsidation of DNA, it is hypothesized that L2 binds to E2 and uses it to traffic the genome of the virus to where virion assembly occurs (Holmgren *et al.*, 2005).

HPV genome replication occurs in the nuclear substructures known as nuclear domain 10 (ND10) (Tavalai and Stamminger, 2008). In natural infection this site serves as the localisation site of expressed L2, which is essential for capsid assembly. Florin *et al.* (2002) worked on the translocation of the structural L1 and L2 proteins of HPV 33 and noted that they are independently translocated into the nucleus where virion assembly happens. This is because both the L1 and L2 proteins have nuclear localization sequences which enable their translocation (Darshan *et al.*, 2004). *In vivo*, translocation of L2 from the cytoplasm precedes that of L1 (Florin *et al.*, 2002). The authors also showed that L2 expression and localization in ND10 precedes L1 accumulation by several hours. Interestingly, Egawa *et al.* (2000) showed that HPV 1 L1 is expressed and located in the nucleus all over the epithelium, whereas L2 was only expressed in the upper epidermal layers

Conway *et al.* (2009) demonstrated that, following capsid assembly, naive virions utilize natural redox gradients in the upper layers of differentiating epithelial cells to facilitate redox-dependent maturation by forming disulfide bonds among neighbouring L1 proteins. Using cell culture derived capsids Buck *et al.* (2005b) showed that papillomavirus capsid maturation is a very slow process that is associated with disulphide bond formation among molecules of L1 protein. Capsid maturation results in more stable capsids that are resistant to proteolytic lysis (Buck *et al.*, 2005b). Interestingly, contrary to the conventional lytic cycle of non-enveloped viruses, it is suggested that papillomaviruses are released by desquamation, a process where keratinocytes lose their structural stability after they are freed from the skin surface (Cardone *et al.*, 2014, Buck *et al.*, 2005b). The authors suggested that, capsids are stabilized through disulfide interactions in the oxidative environment before their release into the environment.

1.4 Native and Synthetic HPV Particles

Virions from organotypic cultures are not yet structurally comparable to those from natural infection, however, their production depends on the differentiation of keratinocytes, which indicates their novelty (Conway and Meyers, 2009). These systems however are time consuming and inefficient in the production of native HPV virions (Conway *et al.*, 2011). The production of synthetic particles (VLPs and PsVs) partially overcame these restrictions (Horvath *et al.*, 2010). This involves the

expression of HPV structural proteins and a reporter gene (in the case of PsVs) in eukaryotic expression systems. The structural and immunological features of these particles are similar to those of the parental HPV virions (Xu *et al.*, 2006).

1.4.1 Virus-like particles

The capsid proteins of many viruses self-assemble into VLPs with similar structural properties as native viruses (Grgacic and Anderson, 2006). Figure 1.6 illustrates the main difference between HPV L1 VLP and authentic virion. Although VLPs mimic native virions, they are not infectious since they do not contain the genetic material of the virus (Figure 1.6) (Deml *et al.*, 2004). Furthermore, these VLPs display high densities of epitopes which often stimulate strong immune responses (Grgacic and Anderson, 2006). This makes them effective vaccine candidates for many viruses. In addition to the absence of the viral genome, VLPs ensure tissue-specificity making them safe vaccine candidates contrary to attenuated or inactivated virus vaccines (Chroboczek *et al.*, 2014). Notably, VLPs can trigger cytotoxic T lymphocyte and cluster of differentiation-4 (CD-4) proliferative responses (Paliard *et al.*, 2000).

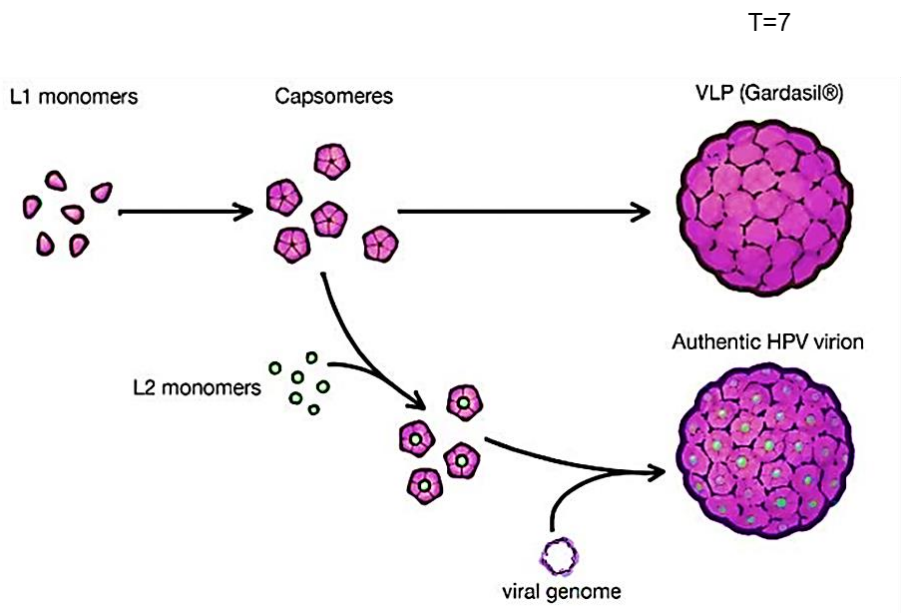


Figure 1.6| Schematic composition of HPV L1 VLP versus native virion: VLPs are structurally like native virion, except that they lack the viral genome. Image adopted from Yu (2017).

HPV VLPs are produced by expressing the major L1 structural protein on its own or together with the minor L2 structural protein. VLPs for vaccine candidates must be

produced in expression systems that are safe, yet yield high production (Noad and Roy, 2003). Traditionally, the inability to propagate HPV *in vitro* as well as the absence of efficient expression systems hindered the development of HPV vaccines (Mach *et al.*, 2006). However, in recent years several methods have been described for successful production of HPV VLPs. The first method produced HPV virions by transfecting mammalian cells with vaccinia viral vector (Zhou *et al.*, 1991). Other methods used insect-cells (Paavonen *et al.*, 2007), yeast (Mach *et al.*, 2006) and bacteria (Chen *et al.*, 2000). Interestingly, HPV VLPs have also been produced in plants (Chabeda *et al.*, 2019, Fernández-San Millán *et al.*, 2008, Maclean *et al.*, 2007, Biemelt *et al.*, 2003). Notably, these particles resemble native virions structurally and immunologically (Biemelt *et al.*, 2003).

Antibodies induced by canine oral papillomavirus (COPV) VLPs protected beagles against experimental oral mucosal papillomas infections (Suzich *et al.*, 1995). Furthermore, cottontail rabbit papillomavirus (CRPV) VLPs protected rabbits against experimentally induced CRPV (Breitburd *et al.*, 1995). Interestingly, HPV VLPs activated dendritic cells *in vitro*, which activates primary immune response against pathogens *in vivo* (Lenz *et al.*, 2001, Rudolf *et al.*, 2001). Most studies demonstrated that, vaccinations with HPV VLPs result in high titres of protective antibodies, thus protecting humans from HPV infections (Ault *et al.*, 2004, Fife *et al.*, 2004, Emeny *et al.*, 2002, Koutsky *et al.*, 2002). VLPs have since been applied as prophylactic vaccines against HPV, they are the foundation on which the current commercially available HPV vaccines have been developed.

1.4.2 Pseudovirions

After the production of HPV VLPs that resemble parental virions, some groups showed that viral L1 and L2 capsids can encapsidate reporter plasmids to generate PsVs that infect cells and tissues (Buck *et al.*, 2005a, Roden *et al.*, 1996). Roden *et al.* (1996) used a Semliki Forest virus vectorto generate PsVs for HPV 16 in hamster BPHE-1 cells, whereas Unckell *et al.* (1997) used the vaccinia viral vector to generate PsVs for HPV 33 in monkey COS-1 cells. The authors noted that the minor L2 structural protein is essential for genome encapsidation.

PsVs can efficiently deliver plasmid DNA into multiple cell lines and tissues, in a similar way to a native HPV virion delivering it’s genome, thus they can be used to measure

protective neutralizing antibody titers *in vitro* or *in vivo* and in medical applications such as gene therapy (Ma *et al.*, 2011). Interestingly, Hung *et al.* (2012) showed that HPV 16 PsVs can successfully deliver herpes simplex virus thymidine kinase (HSV-tk) gene to ovarian tumour cells, resulting in significant therapeutic anti-tumor effects in mice. This suggest that HPV PsVs can be utilised as a potential gene delivery system in gene therapy.

Apart from failure to propagate HPV in tissue culture, the absence of effective neutralizing assays has been a major drawback in the production of vaccines against HPV infections. The development of PsVs has enabled researchers to measure neutralization of HPV infectivity both *in vivo* and *in vitro*. Subsequently, PsVs are used in PBNAs (Buck *et al.*, 2005a, Pastrana *et al.*, 2004, Yeager *et al.*, 2000). PBNAs are considered as standard assessment for the immunogenicity of HPV candidate vaccines (Lamprecht *et al.*, 2016). However, PsVs are currently made in human embryonic kidney cells (HEK293TT cells) which is a highly expensive system. This might be a major drawback in the development of affordable HPV vaccines for developing country and a cost-effective alternative would be good. In an attempt to reduce the high expenditure associated with PsVs production in mammalian cells, Lamprecht *et al.* (2016) successfully produced PsVs in plants with comparable characteristics to mammalian produced PsVs.

In *in vitro* standard assays, HPV L2 based vaccines yield very low level of neutralising antibodies (Day *et al.*, 2012), although they effectively protect animals against experimental challenges (Gambhira *et al.*, 2007a, Palmer *et al.*, 2006, Embers *et al.*, 2002). Therefore, there is a possibility that standard neutralization assays are not a sensitive measurement for HPV L2 based vaccines infectivity, this led to the development of L2-specific PBNA. This assay development was based on the understanding of initial steps that happen on the basement membrane during viral infection, particularly the conformational change that expose cross-neutralization epitopes of L2 molecule (Day *et al.*, 2012).

1.5 Detection of HPV infections

Cervical cancer deaths have been reduced in developed countries due to timely screening and intervention. Screening of cervical cancer has essentially been based on the cost-effective cytology-based method described by George Papanicolaou

known as the Papanicolaou test, or “Pap smear” (Papanicolaou and Traut, 1941). The method detects early cervical lesions, and have successfully reduced the cervical cancer mortality rate, especially in developed countries (Behtash and Mehrdad, 2006, Levi *et al.*, 2000).

However, false-negative results have been reported in Pap smears as the efficacy of the test largely depends on the sample quality (Cuzick *et al.*, 2006, O'Meara, 2002). Since then, research have focused on the development of screening technologies that will complement and improve the cytology-based methods. Subsequently, novel diagnostic tools for detecting HPV infections have been established, these include: detection of carcinogenic HPV DNA with polymerase chain reaction (PCR)-based methods, the use of epithelial cell markers to detect HPV early and/or late proteins, and finally serological markers to detect HPV antibodies in serum and other body fluids (Pagliusi and Garland, 2007).

PCR-based screening of cervical cancer by targeting HPV DNA have demonstrated high sensitivity for cervical intraepithelial neoplasia (CIN) than that achieved by cytology-based method (Cuzick *et al.*, 2006). However, factors such as age at which to begin cervical screening may negatively influence reduction of cervical cancer using HPV DNA testing methods (Ylitalo *et al.*, 2000, Cuzick *et al.*, 2006). On the other hand, the immunological detection of HPV in human cells and tissues is being hindered by 3 main factors. These are: low expression level of HPV early proteins, expression of the E6 and E7 proteins only in the productive phase, and lack of quality antibodies with high sensitivity against the viral proteins (Pagliusi and Garland, 2007).

Serological assays for screening HPV antibodies in sera and other body fluids have been described, although the diagnostic value for HPV detection in other body fluids is yet to be established. Monoclonal antibodies that can identify and characterize different HPV neutralizing epitopes have been developed (Christensen *et al.*, 1996b). However, these antibodies are not commercially available and currently made in mice and are costly. Costly production of these antibodies may potentially restrict their use in developing countries. Therefore, there is a need to develop HPV antibodies using an affordable system that can be used in diagnostics and research.

1.6 HPV vaccines

1.6.1 Prophylactic HPV vaccine

1.6.1.1 Licensed HPV prophylactic vaccines

Generally, vaccination is the most cost effective and successful intervention in preventing infectious diseases (Kushnir *et al.*, 2012, Daniell *et al.*, 2009). Traditionally, viral vaccines were either attenuated or inactivated virions. These vaccines have been historically effective, such that they have led to the eradication of target pathogens, for example smallpox in humans. However, live attenuated vaccines can cause side effects especially in immune-compromised individuals (reviewed in Plotkin (2005)). This led to the establishment of other alternative approaches, to develop effective yet safe vaccines.

A major advance in recombinant DNA technology and genetic engineering was the establishment of sub-unit vaccines in 1988 (Murray, 1988). Specifically the usage of VLPs to produce safe sub-unit vaccines with enhanced immunogenicity (Kushnir *et al.*, 2012). Currently, there are vaccines in the market that are VLP-based like Sci-B-Vac™ for Hepatitis B virus (HBV); and Gardasil®, Cervarix™ and Gardasil9® for HPV. Currently, plant-made VLP-base vaccine against Avian H5N1 Influenza by Medicago Inc is phase III of human clinical trials (Pillet *et al.*, 2019). Furthermore, Mosquirix™ developed by GlaxoSmithKline for malaria was approved by European regulators (Hawkes, 2015).

Just like any other infection, the recognition of HPV infection as the etiological agent of cervical cancer motivated researchers to focus on the production of HPV vaccines. The major L1 structural protein has the potential to self-assemble into VLPs that are structurally and immunogenically like native virions (Mach *et al.*, 2006), which is the foundation on which current commercially available HPV vaccines have been developed.

Currently, there are 3 commercially available prophylactic VLP-based HPV vaccines which elicit neutralizing antibodies. These vaccines are all formulated in aluminum-based adjuvants and are generally known to be highly effective and safe (Joura *et al.*, 2015, Clark *et al.*, 2013, Mesher *et al.*, 2013). In 2006, the United States of America (USA) Food and Drug Administration (FDA) approved the first HPV vaccine for use in

humans: Gardasil® a quadrivalent HPV 11/18/6/16 L1 VLP vaccine by Merck and Co. Inc, which protects against HPV 6, 11, 16 and 18. Additionally, in 2009 the FDA approved a second vaccine, the bivalent HPV 18/16 VLP vaccine, Cervarix™ developed by GlaxoSmithKline. This vaccine protects against HPV 16 and 18 infections.

Both Gardasil® and Cervarix™ are efficacious against their corresponding HPV types (Paavonen *et al.*, 2009, Garland *et al.*, 2007, Harper *et al.*, 2006). Interestingly, there is cross-protection of HPV 31, 33 and 45 with Cervarix™, and HPV 31 with Gardasil® (Paavonen *et al.*, 2009, Wheeler *et al.*, 2009, Harper *et al.*, 2006). However, the authors noted that the strength of the cross-neutralizing antibodies are lower than those of the targeted HPV types.

In 2014 the FDA approved yet another vaccine, Gardasil9®, a nonavalent HPV 6/11/16/18/31/33/45/52/58 vaccine. Gardasil9® has a high L1 antigen load, and has the potential to prevent 90% of anal, vulvar, vaginal and cervical cancers (Printz, 2015). However, the worldwide distribution and occurrence of HPV types differ per geographic regions with Africa having the highest occurrence of ~21.1-29% of HPV infections (Zhai and Tumban, 2016). Therefore, the vaccine might have different success rates in different regions. For example, in Africa where the highest burden of cervical cancer is reported, HPV 35 is more prevalent and important than other HPV types included in the current vaccines (Smith *et al.*, 2007c). Unfortunately, HPV 35 in not included in any of the commercially available vaccines.

The government of some countries such as Australia and the USA have introduced national HPV vaccine programmes with Gardasil9®. In fact, by 2017 and 2018 Gardasil9® was the only HPV vaccine available for distribution in the USA and Australia, respectively (Markowitz *et al.*, 2018). Gardasil9® is well tolerated, highly immunogenic and efficacious against high-grade cervical and cytological abnormalities caused by HPV types included in the vaccines (Garland *et al.*, 2018, Ruiz-Sternberg *et al.*, 2018, Huh *et al.*, 2017).

Although these vaccines are effective at preventing HPV infection, they are type-restricted, meaning they offer protection to a restricted number of HPV types (Matić *et al.*, 2012). Therefore, second-generation HPV preventative vaccines that will produce a wider spectrum protection against multiple HPV oncogenic types need to be

developed. Furthermore, these vaccines are produced in highly expensive expression systems. Gardasil® and Gardasil9®, are produced in the *Saccharomyces cerevisiae* yeast expression system, whereas Cervarix™ is produced in insect-cells (Hi-5-Rix4446 derived from *Trichoplusia ni*) (Palmer *et al.*, 2009).

Due to high cost associated with the commercially available HPV vaccines, most low-income countries are not yet ready to introduce HPV vaccination programmes, with only 7 out of 54 African countries having vaccination programmes as of May 2018 including South Africa (Chido-Amajuoyi *et al.*, 2019). Only Cervarix™ and Gardasil® are currently available for distribution in Africa, which only protect against few oncogenic HPV types. In South Africa Gardasil® is available in private sectors, whereas Cervarix™ is currently administered to girls aged ≥ 9 years, which is part of the national HPV vaccine programme that was introduced in 2014 (Delany-Moretlwe *et al.*, 2018). Consequently, there is a need to develop affordable multivalent HPV vaccines for use especially in developing countries, where cancer of the cervix is the greatest burden with a reported 87% of the global HPV infection (Ferlay *et al.*, 2015).

1.6.1.2 Second generation prophylactic vaccines

1.6.1.2.1 L2-based vaccines

L1 is known to be more immunodominant than L2 (Palmer *et al.*, 2009). As a result, several groups successfully produced HPV L1 VLPs (Chabeda *et al.*, 2019, Maclean *et al.*, 2007, M'hirsi El Adab *et al.*, 2007, Warzecha *et al.*, 2003, Kirnbauer *et al.*, 1992). Research based on highly immunogenic HPV L1 VLPs led to FDA approval and commercial sales of VLP-based vaccines. However, the unaffordability and the type-specificity of these vaccines and their poor cross protection of heterologous HPV types are the major drawback of these vaccines.

In an effort to overcome these challenges, specifically the poor cross-protection against heterologous HPV types, early efforts discovered that the amino terminus of the minor L2 structural protein comprises epitopes capable of inducing protective neutralizing antibodies against HPV (Campo *et al.*, 1997, Knowles *et al.*, 1997, Chandrachud *et al.*, 1995). Regardless of this discovery, recombinant L2 demonstrated poor immunogenicity compared to L1 VLPs.

Few groups demonstrated that the HPV L2 amino terminus can trigger neutralizing antibodies that can defend against specific HPV types and cross-neutralize other HPVs (Slupetzky *et al.*, 2007, Palmer *et al.*, 2006, Pastrana *et al.*, 2005, Roden *et al.*, 2000) . This directed researchers to focus on HPV L2-based vaccines as alternatives to types-specific L1 based vaccine candidates. Particularly to locate L2 sequences that can cross-neutralize other papillomavirus infections (Alphs *et al.*, 2008, Gambhira *et al.*, 2007b).

In vivo immunogenicity of a distinct region of L2 was demonstrated using rabbits (Embers *et al.*, 2002). This group immunized rabbits with synthetic peptides each containing short peptides of L2 from either CRPV or rabbit oral papillomavirus (ROPV) and subsequently challenged rabbits with CRPV and ROPV. Both oral and cutaneous papilloma growths were inhibited in rabbits immunized with the ROPV and CRPV peptides, respectively.

Roden *et al.* (2000) demonstrated cross neutralization of heterologous HPV types by L2 polypeptides of HPV 18, 6 and 16. Additionally, Palmer *et al.* (2006) used recombinant tobacco mosaic virus (TMV), comprising CRPV L2 epitopes to successfully protect rabbits from experimentally induced papillomavirus with cross-protective immunity. Slupetzky *et al.* (2007) further demonstrated the cross-protection ability of HPV 16 L2 epitopes against HPV 11. From all these data it is evident that L2 epitopes might be a good target for the development of broad-spectrum protection. L2 induced neutralizing antibody titres are however usually 2-3 orders of magnitude less than L1-induced titres (Pastrana *et al.*, 2005).

1.6.2 Therapeutic HPV vaccines.

Commercially available prophylactic vaccines are effective at preventing HPV infections, however there is a high occurrence of established HPV infections globally which current vaccines are ineffective at treating (Kim *et al.*, 2014, Lin *et al.*, 2010, Schiller *et al.*, 2008). Current vaccines are designed to induce neutralizing antibodies by targeting HPV structural proteins (L1 and L2). However, these capsid proteins are not detectable in HPV infected cells after primary infections (Schiller *et al.*, 2008). Epithelial cells infected with HPV present non-capsid proteins in the context of major histocompatibility complex (MHC) class I to the immune system (Frazer, 2009). Therefore, to effectively treat existing infections and associated lesions, therapeutic

HPV vaccines are required to induce antigen-specific cellular immune responses against cells infected with HPV (Lin *et al.*, 2010, Frazer, 2009).

To date, HPV therapeutic vaccines are designed to use the early genes E6 and E7 as antigens because they are tumour-specific, and are not self-protein hence there are no concerns of autoimmunity (Lin *et al.*, 2010, Hung *et al.*, 2008a). Furthermore, they are involved in cell disruption and are expressed constitutively in cervical cancers and premalignant lesions (Zur Hausen, 2002). Currently, there is no approved therapeutic HPV vaccine. However, several approaches have been studied with the hope of developing effective HPV therapeutic vaccines and are in clinical trials. These strategies include peptide and protein vaccines, vector-based vaccines, and nucleic acid vaccines. Others such as whole-cell based vaccine, combinational approaches and prime-boost vaccination are also being studied.

Peptide vaccines can be either: long peptides or short (specific epitopes) peptides. These vaccines are favoured because they are stable, safe and easy to develop (Hung *et al.*, 2008b). However, their antigens are limited to a major histocompatibility complex (MHC) class I phenotype that is expressed by the patient (Su *et al.*, 2010, Lin *et al.*, 2010). Furthermore, their immunogenicity is very low and must be administered with co-stimulatory molecules or adjuvants like Toll-like receptor (TLR) ligands (Yang *et al.*, 2016, Lin *et al.*, 2010). Nonetheless, with adjuvants, peptide-based vaccines such as a synthetic long-peptide HPV 16 vaccine in incomplete Freund's adjuvant and four HPV 16 synthetic peptide vaccines with Candida skin test reagent (PepCan) made it to clinical trials with significant cell mediated immune responses in cervical cancer patients (Coleman *et al.*, 2016, Kenter *et al.*, 2009). The utilization of peptide-based vaccines therefore depends on the improvement of their immunogenicity and antigen presentation (Bolhassani, 2018).

Protein-based vaccines overcame the challenge of MHC class I molecule specificity because of the various number of epitopes, however they still need adjuvants due to their low level of immunogenicity (Lin *et al.*, 2010). Additionally, their poor immunogenicity may induce humoral rather than cell-mediated response (Su *et al.*, 2010). HPV protein-based vaccines have also advanced to clinical trials with the help of adjuvants and were noted to mount specific immune responses (Daayana *et al.*, 2010, Roman *et al.*, 2007, Davidson *et al.*, 2004, Frazer *et al.*, 2004, Hallez *et al.*,

2004). Notably, in a phase II trial, tissue antigen-cervical intraepithelial neoplasia vaccine (TA-CIN), has been shown to induce both the antibody and cellular (T-cell specific) mediated responses (Daayana *et al.*, 2010). Furthermore, HPV-16 E7 encoding a heat shock protein (Hsp) fusion protein vaccine (SGN-00101) induced lesion regression in correlation with immune responses in women with high-grade CIN (Roman *et al.*, 2007). Interestingly, a plant-made HPV 16 therapeutic candidate vaccine (16E7SH) by our group showed significant cell-mediated immune response and tumor regression in mice (Whitehead *et al.*, 2014). These data indicate that improving the immunogenicity of protein-based vaccine may lead to rapid eradication of lesion. Therefore, enhancing the immunogenicity of protein-based vaccine is the key to the usage of these vaccines.

Researchers have also looked at vector vaccines, which are based on the modification of non-pathogenic viruses or bacteria that replicate inside the host cell and express antigens. Vectors like adeno-associated viruses (AAVs), vaccinia virus and *Listeria monocytogenes* have been used in the development of these vaccines. A single dose of recombinant AAV type 5 encoding HPV 16 L1 induced sufficient mucosal antibodies in vaginal washes and L1 specific serum antibodies in mice (Kuck *et al.*, 2006). Interestingly after 60 weeks of immunization, cellular immunity was still detectable. Using a vaccinia-based vaccine, HPV 16 E2- based vaccine effectively prevented HPV growth in high grade lesions (Rosales *et al.*, 2014). Recombinant *L. monocytogenes* expressing HPV 16 E7 caused tumor growth regression in mice (Lin *et al.*, 2002). However, there are concerns of safety, as these vaccines may pose danger in immunocompromised individuals (Lin *et al.*, 2010).

DNA vaccines have been extensively studied and gained traction as a foundation for HPV therapeutic vaccines. DNA vaccines are easy to manufacture, safe, stable and cause antigen-specific immunity (Lin *et al.*, 2010, Gurunathan *et al.*, 2000). Furthermore, DNA vaccines are not restricted to class I MHC molecules due to multiple epitopes and have unmethylated CpG motifs to act as adjuvants (Lin *et al.*, 2010). The low level of immunogenicity has been improved by several strategies including the improvement of HPV antigen processing, expression and presentation in dendritic cells (DC), and increasing the interaction between the DC and T cells (Tsen *et al.*, 2007). HPV DNA therapeutic vaccines such as pNGVL4a-CRT-E7(detox) and E6/E7 vaccine known as GX-188E, advanced to clinical trials with robust cellular immune

response (Alvarez *et al.*, 2016, Kim *et al.*, 2014). Therapeutic vaccines are a promising approach to cancer treatment. Currently therapeutic vaccines are designed to induce antigen-specific cellular immunity and various clinical trials are underway to determine effective and safe vaccines.

1.7 Plant expression systems

Generally, the unaffordability of vaccines is due to their complex production systems (Daniell *et al.*, 2009). Over the years, plants have gained traction as a cost-effective alternative to conventionally used expression systems (yeast, mammalian cells, bacteria and insects). Plant production platforms are rapid, affordable, easy to use and safe as they lack mammalian pathogens and toxins (Kushnir *et al.*, 2012, Rybicki, 2010, Fischer *et al.*, 2004). Furthermore, plants have the eukaryotic machinery required for protein modification and folding (Fischer *et al.*, 2004).

Plant expression systems have been utilised since 1986, after a human growth hormone was produced in stably transformed tobacco and sunflower callus tissue (Barta *et al.*, 1986). Shortly after, Hiatt *et al.* (1989) successfully produced a full monoclonal antibody in transgenic plants. Hepatitis B surface antigen was the first plant produced vaccine candidate (Mason *et al.*, 1992). Since then various vaccine antigens have been effectively produced in plants. One of the recent breakthroughs in plant production is the quadrivalent VLP-based influenza vaccine candidate by Medicago Inc that is currently in the first phase III human trial. Data from phase II clinical trials demonstrated that the vaccine is safe and induced consistent humoral and cellular immune responses in adults (Pillet *et al.*, 2019). The authors further noted that the vaccine is expected to elicit strong immune response in phase III clinical trial, data from this trial is expected to be available in coming months.

Notably, in May 2012 the first plant-based enzyme replacement therapy was approved by the FDA for commercial use in humans (Fox, 2012). The enzyme, known as Elelyso™, is a recombinant glucocerebrosidase (GCD) used in Gaucher's disease treatment and is produced in transgenic carrot cells by Protalix Biotherapeutics and Pfizer (Carmiel, Israel). Gaucher is a lysosomal storage disorder (LSD) triggered by a lack of the GCD enzyme (Shaaltiel *et al.*, 2007).

Interestingly, during the 2014 West Africa Ebola outbreak, triple cocktail (2G4, 4G7, 13C6) expressed in *N. benthamiana* was FDA approved for use to contain the Ebola

virus outbreak (Qiu *et al.*, 2014). Furthermore, there are various plant produced antibodies, therapeutic proteins and vaccines that are in clinical development including Intrinsic factor for vitamin B12 deficiency, and Norwalk virus coat protein (CP) (reviewed in Yusibov *et al.* (2011)). All these data suggest that plant expression systems have the potential to develop affordable vaccines especially for use in developing countries.

Plant expression systems are divided into two methods: transgenic or transient expression systems (Scotti and Rybicki, 2013). Traditionally, plant expression approaches relied on stably transformed plants, a genomic incorporation of a transgene into the plastid or nuclear genome of the plant (Schillberg *et al.*, 2005). Transgenic plants are obtained through gene delivery into monocots by means of biolistic or into dicots by *Agrobacterium tumefaciens* (*A. tumefaciens*) (Li *et al.*, 1993, Mason *et al.*, 1992). However, generating transgenic plants is a time consuming process (Huang *et al.*, 2010, Schillberg *et al.*, 2005).

Transient expression of recombinant proteins recently became an alternative to transgenic expression systems. In contrast to stably transformed plants, transient expression systems can rapidly yield high levels of proteins in a very short time (Rybicki, 2010, Schillberg *et al.*, 2005). Plant-based transient expression of heterologous recombinant proteins is accomplished by either the infection of plants with viral vectors from cowpea mosaic virus (CPMV) , Potato X virus (PVX) or tobacco mosaic virus (TMV) or by infiltration with recombinant *Agrobacterium* (agroinfiltration) (Varsani *et al.*, 2006, Kapila *et al.*, 1997). With the viral vector approach, the gene of interest is cloned into the genome of the plant virus, with a strong subgenomic promoter. The resulting recombinant virus construct is then used to infect plants and deliver the transgene to the cell to yield the desired protein without having to stably transform the plant (Porta and Lomonossoff, 2002). Although this approach produced immunogenic protein, the expression levels of the protein of interest were relatively low compared to the transgenic plants (Turpen *et al.*, 1995).

These drawbacks caused the establishment of a new ideal strategy of deconstructed virus vectors and agroinfiltration (Marillonnet *et al.*, 2005, Gleba *et al.*, 2005). Altered plant viruses such geminiviruses, tobomaviruses, bromoviruses and bean yellow dwarf mastrevirus are commonly used in this approach. This approach uses the viral

replicative machinery, to synthesize non-infectious vectors that can induce transgene expression in plants (Gleba *et al.*, 2005). In our group, viral vectors such as pRIC, pTRA vector suit and pEAQ-*HT* are used to transiently express pharmaceutically relevant recombinant proteins in plants (Chabeda *et al.*, 2019, Gunter *et al.*, 2019, Pineo *et al.*, 2013, Regnard *et al.*, 2010, Sainsbury *et al.*, 2009, Maclean *et al.*, 2007).

Employing such vectors together with agroinfiltration results in higher levels of protein expression in a short period of time (3-7 days) compared to 6-9 months when using stably transformed plants (Tiwari *et al.*, 2009, Gleba *et al.*, 2005). Therefore, transient expression mediated by agroinfiltration via viral vectors is an ideal and cost-effective model for proteins production in plants (Rybicki, 2010). Notably, there is great potential to reduce high expenditure production of HPV VLPs and PsVs by using transient expression system (Santi *et al.*, 2006).

1.7.1 HPV VLPs and PsVs production in plants

HPV VLPs have effectively been expressed in transgenic plants (Biemelt *et al.*, 2003, Varsani *et al.*, 2003, Warzecha *et al.*, 2003). However, yields of VLPs were very low. Furthermore, early efforts to transiently express HPV VLPs in plants also resulted in very low expression levels and with mainly capsomeres and few VLPs (Varsani *et al.*, 2006).

The work done by Varsani *et al.* (2006) laid the groundwork for further HPV research, particularly the production of VLPs in plants. Maclean *et al.* (2007) looked at plant, and human codon-optimization of HPV L1 gene and targeting of different cell compartments. The authors noted, high level of recombinant protein expression with fully assembled VLPs when using a human codon-optimized L1 gene as compared to plant codon-optimized L1 gene. Furthermore, protein expression was high when using a chloroplast-targeting vector than a cytoplasm-targeting vector. Their work demonstrated that plant transient-based expression system can be used to produce HPV VLPs. Utilizing the same approach Chabeda *et al.* (2019) and Pineo *et al.* (2013) successfully produced HPV 16 immunogenic L1 and L1/L2 chimaeric VLPs in plants. In both cases HPV16 L1 and L1/L2 chimaeric proteins were expressed at high levels using the pTRA-CTP vector, a non-replicating plant expression vector that targets the protein to the chloroplast.

PsVs have successfully been produced in *N. benthamiana* using human codon-optimized L1 and L2 genes and a secreted alkaline phosphatase (SEAP) reporter plasmid (Lamprecht *et al.*, 2016). The authors demonstrated that plant-produced HPV 16 PsVs can be used in PBNA with comparable results to PsVs produced by mammalian cells. This is a proof concept that plants may be used as a cost-effective alternative for PsV production. In conclusion, plants can potentially be used to develop safe, effective and affordable HPV vaccines especially for use in developing countries (Lamprecht *et al.*, 2016).

1.8 Rationale and objectives of the study

Cervical cancer is a burden in developing countries with the highest prevalence reported in Africa. However, the costly production of commercially available HPV prophylactic vaccines is limiting their use in these countries. Furthermore, worldwide geographic distribution of HPV types differs, therefore the commercially available HPV vaccines will probably benefit some regions more than others. For instance, HPV 35 is not available in any of the vaccines and is more dominant and important in Africa than other HPV types included in the current vaccines. Therefore, the current vaccines are partially ill-suitable for some regions. Ultimately, there is not only an urgent need to develop affordable HPV vaccines for use in developing countries, but these vaccines also need to address/include all carcinogenic HPV types that are important to these regions. Plants are a cost-effective alternative to widely used expression systems and have demonstrated their potential to make feasible and highly immunogenic vaccines with some FDA approved vaccines and some in human trials. Therefore, making multivalent HPV vaccines in plants will reduce the cost associated with current vaccines, and will particularly benefit resource-poor regions.

This study aimed to evaluate the plant-based transient expression system as a tool to develop multivalent prophylactic HPV vaccines for use in developing countries, especially Africa which has the highest burden of cervical cancer. Furthermore, the study aimed to assess whether plant-produced VLPs can induce humoral responses in mice as effectively as the commercially available Gardasil® and if they are suitable prophylactic HPV vaccine candidates. To reduce the number of animals used in this study, only 3 HPV types that are important in Africa and are not included or partially protected by Gardasil®, were selected to immunize mice.

Specifically, the objectives of the study were as follows:

1. To transiently express the L1 proteins of 8 most common high-risk HPV types in Africa, and 2 low-risk types (HPV 6, 16, 18, 31, 33, 34, 35, 45, 52 and 58) in *N. benthamiana.*
2. Purification of putative plant-produced L1 VLPs proteins by density gradient centrifugation and TEM to determine if the expressed L1 proteins successfully assembled into VLPs.
3. To vaccinate mice with selected plant-produced VLP vaccine candidates (produced in Objective 2) and Gardasil® (positive control) and to analyse the immunogenicity elicited by the vaccine candidates using ELISAs and to determine L1 neutralizing antibodies using the standard L1 PBNAs.

2 Chapter 2: Transient expression and purification of HPV L1 VLPs in *Nicotiana* benthamiana

2.1 Introduction

The HPV L1 capsid protein can self-assemble into VLPs that are structurally and immunogenically similar to native virions (Jordan *et al.*, 2019, Chabeda *et al.*, 2019, Biemelt *et al.*, 2003, Hagensee *et al.*, 1993, Zhou *et al.*, 1991). Interestingly, these VLPs can efficiently induce T-cell responses due to their particulate nature (Mohsen *et al.*, 2019, Mohsen *et al.*, 2018, Pineo *et al.*, 2013). Furthermore, their surface has arrays of repetitive epitopes which are recognised by B cells, making these VLPs highly immunogenic compared to other sub-unit vaccines (Giannini *et al.*, 2006). Notably, 3 HPV prophylactic vaccines based on L1 VLPs are commercially available and are effective at preventing HPV infections.

HPV VLPs are produced in various systems, including: bacteria, yeast, insect-cell, mammalian and plants expression systems (Chabeda *et al.*, 2019, Pineo *et al.*, 2013, Maclean *et al.*, 2007, Biemelt *et al.*, 2003, Christensen *et al.*, 1994, Kirnbauer *et al.*, 1992, Zhou *et al.*, 1991). In bacterial expression systems, mostly *Escherichia coli* (*E. coli*), the virus capsid protein genes are codon-optimized for *E. coli* expression and are cloned in plasmids under strong promoters for increased protein expression (Brown *et al.*, 2009b, Chen *et al.*, 2000). HPV VLPs are produced by over-expressing the major L1 capsid protein in bacterial cells, followed by *in vitro* assembly/disassembly of purified L1 (Chen *et al.*, 2001, Chen *et al.*, 2000, Touzé *et al.*, 2000). A disadvantage of using bacterially-produced VLPs is a safety concern as the recombinant proteins might contain endotoxins (Chen and Lai, 2013, Roldão *et al.*, 2010, Grgacic and Anderson, 2006).

For production of the commercially available HPV L1 VLP-based vaccines; Gardasil®, Gardasil9® and Cervarix™, yeast and insect cells, respectively are used. These vaccines are produced by subjecting purified L1 to *in vitro* disassembly/reassembly treatments, which cause VLPs assembly and improve antigenicity, stability and structural integrity of VLPs (Zhao *et al.*, 2014). These additional steps increase the already high expenditure of the overall production of VLPs in these systems (Chen and Lai, 2013). Mammalian expression systems are another ideal choice to produce VLPs as they enable post-translational modifications, which is crucial for the correct

folding of VLPs. , However this is a highly expensive system compared to other systems (Lai and Chen, 2012, Chen, 2008).

Preventing HPV infections through vaccination significantly reduced cervical cancer death in developed countries. However, due to high cost associated with current HPV vaccines, this is still just a dream in developing countries (Mohsen *et al.*, 2019, Waheed *et al.*, 2016). Generally, the high costs of these vaccines are due to their complex production systems. Therefore, cost-effective alternative HPV vaccine production platforms need to be established for use in resource-poor countries. Over recent years, plants have gained traction as cost-effective alternative production systems for recombinant proteins

Plant production platforms are safe, robust, highly scalable, with low production costs and have the eukaryotic machinery required for proper post translational modifications and assembly (Rybicki, 2010, Fischer *et al.*, 2004). Therefore, using plant platforms to produce vaccines might overcome the disease burden in developing countries.

Plant expression systems can either be transgenic or transient. Recently, transient expression system gained attraction over stable transformed plants. This is because transient expression is in itself a contained system and can be used to rapidly produce large amount of proteins, thereby saving time (Marsian and Lomonossoff, 2016, Fischer *et al.*, 2012, Fischer *et al.*, 2004). Transient expression is commonly achieved by introducing recombinant A*grobacteria* harbouring a plant expression vector containing the gene of interest into plant leaves, mainly by syringe or vacuum infiltration, in a process known as agroinfiltration (Suzaki *et al.*, 2019, Hoshikawa *et al.*, 2019, Musiychuk *et al.*, 2007). The process relies on the ability of *A. tumefaciens* to transfer the T DNA from the tumor inducing (Ti) plasmid into the cell nucleus of the plant (Tzfira *et al.*, 2004).

Studies that aimed to produce affordable HPV vaccines in plants mainly focused on the high-risk HPV 16 L1 and/or L1/L2 chimaeras (Chabeda *et al.*, 2019, Zahin *et al.*, 2016, Pineo *et al.*, 2013, De la Rosa *et al.*, 2009, Fernández-San Millán *et al.*, 2008, Maclean *et al.*, 2007, Varsani *et al.*, 2006, Liu *et al.*, 2005, Biemelt *et al.*, 2003, Varsani *et al.*, 2003) and low-risk HPV 11 L1 (Kohl *et al.*, 2007, Warzecha *et al.*, 2003), which have been shown to elicit strong immune responses in animals. This gave rise to this study to further investigate if VLPs for other important oncogenic HPV types could be

produced in plants. In this study, the production of affordable HPV vaccines in plants was of specific interest, with the focus on the oncogenic HPV types that are important in Africa.

Figure 2.1 shows the prevalence of 8 most frequent high-risk HPVs in the world (A) and in Africa (B). HPV 35 is not included in any of the commercially available HPV vaccines and is more dominant and common in Africa. Worldwide, HPV 35 is the 8th most dominant high-risk HPV, while in Africa it is the 5th (Figure 2.1A and B respectively) (Smith *et al.*, 2007c). Although relevant in an African context, HPV 16 and 18 are included in Gardasil®, whereas high-risk HPV 31 is cross-protected by Gardasil® which is available for distribution in Africa (Brown *et al.*, 2009a). For the remining HPV types (33, 45, 52 and 58), HPV 52 and 58 are among the most frequently reported high-risk in Sub-Saharan Africa (McDonald *et al.*, 2014, Akarolo-Anthony *et al.*, 2013, Gage *et al.*, 2012, Veldhuijzen *et al.*, 2011). In Africa, HPV 58 and HPV 52 are ranked 7th and 8th most prevalence high-risk HPVs, respectively (Figure 2.1B) (Smith *et al.*, 2007c).

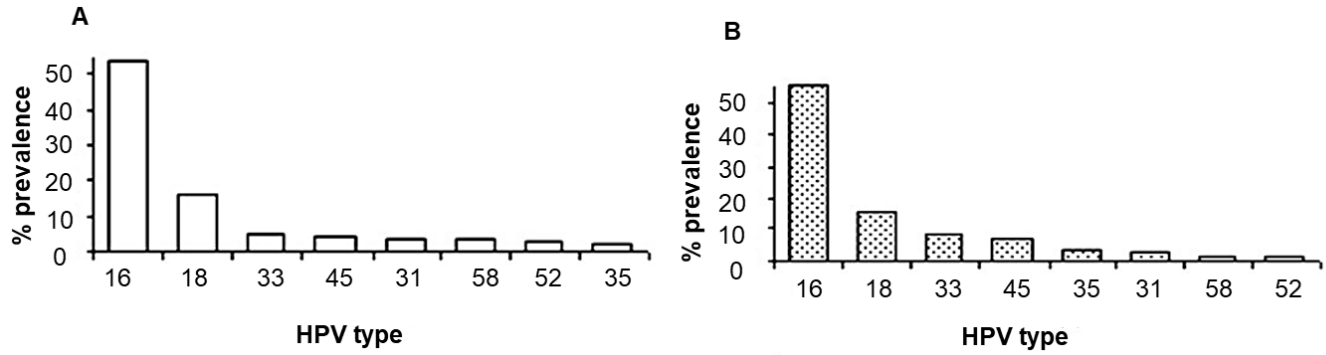


Figure 2.1: Prevalence of 8 most common high-risk HPVs: A) Globally B) Africa. Data are based on 14 500 cervical cancer cases with 1 340 cases from Africa. Figure obtained with permission from Smith *et al.* (2007c).

In an effort to develop a cost-effective HPV vaccine candidate, the L1 proteins of 8 most frequent high-risk HPVs in Africa (HPV 16,18, 31, 33, 35, 45, 52, 58) (Figure 2.1B) and 2 low risk types (HPV 6 and 34) were transiently expressed in *N. benthamiana.* Following successful expression of all the 10 HPV types in plants, 3 oncogenic HPV types (HPV 35, 52 and 58) were selected to immunize mice and were quantified and prepared for animal studies (Chapter 3). Only 3 HPV types, that are relevant in an African context and are not included in Gardasil® or cross-protected by

this vaccine were selected for animal studies to minimize the number of animals used in this study.

In conclusion, the L1 proteins of 10 HPV types were transiently expressed in *N. benthamiana*. Proteins were purified via isopycnic ultracentrifugation using sucrose and Optiprep™ density gradients, and the formation of VLPs assessed by TEM. HPV 35, 52 and 58 VLP vaccine candidates were quantified and prepared for animal studies (chapter 3).

2.2 Materials and methods

2.2.1 Bacterial strains

Chemically competent *E. coli* DH5α (E. cloni®, Lucigen) and *A. tumefaciens* GV3101::pMP90RK were used in this study. All *E. coli* cultures were grown overnight at 37°C with agitation in Luria-Bertani medium (LB; 1% sodium chloride [NaCl], 0.5% yeast extract and 1% tryptone) according to Sambrook *et al.* (1989). Recombinant *E. coli* were cultured in the presence of 100µg/ml ampicillin to maintain selection pressure. *A. tumefaciens* was cultured in LB enhanced with 10mM 2-morpholineethanesulforic acid (MES) and grown at 27°C with agitation. Transformants select on solid media plates were grown 2-3 days. For liquid cultures glycerol stocks were inoculated into 10ml LB supplemented with the relevant antibiotics and grown for overnight. This 10ml was used as inoculum for 50ml, which was in turn used to inoculate 500ml for large scale infiltrations. Recombinant *A. tumefaciens* constructs were selected and cultured in the presence of 30µg/ml kanamycin, 50µg/ml carbenicillin and 50µg/ml rifampicin. All bacterial cultures were preserved in a final concentration of 25% glycerol and stored at -80°C.

2.2.2 Extraction, and purification of plasmid DNA; restriction enzyme digest and ligation

In this study, all plasmid DNA extractions were isolated using the QIAprep® Spin Miniprep kit (Qiagen). Whereas, all DNA gel purifications were carried out using the QIAquick® Gel Extraction kit (Qiagen). Restriction enzymes and DNA ligase (T4) were from Fermentas (Thermo Fisher Scientific). The procedures of these experiments were all carried out as per the manufacturer's instructions.

2.2.3 Polymerase chain reaction and agarose gels

All PCR reactions used to identify recombinant constructs were carried out using vector (pTRACTP) specific primers provided our laboratory (lab) (Table 2.1).

Table 2.1: Primers used to confirm HPV constructs

Primer name	5'-3' primer sequence	Length (bp)	Tm (°C)
pTRA-F	CATTTCATTTGGAGAGGACACG	22	64
pTRA-R	GAACTACTCACACATTATTCTGG	23	64

In this study, all PCR reactions were carried out using ampliqon Taq DNA polymerase 2x Master Mix Red (Thermo Fisher Scientific) and performed as stated in the manufacturer's protocol, using 0.2µM of each primer and 2mM $MgCl_2$. The reactions were carried out as follows: 1 cycle at 95°C for 5 minutes (denaturing step), then 30 cycles at 95°C for 30 seconds (denaturing step), followed by annealing step at 59°C 30 seconds and then extension step at 72°C for 30 seconds. An additional elongation step was carried out at 72°C for 5 minutes. No DNA template was used as a PCR negative control. All amplified PCR products were separated on 1% agarose gels, with O'GeneRuler™1kb DNA ladder (Thermo Fisher Scientific).

2.2.4 Recombinant HPV plasmids

All HPV L1 genes used in this study were synthesized by GenScript and were human codon optimized. *A. tumefaciens* GV3101::pMP90RK transformed with pTRACTP (used as negative control), a vector targeting expression of proteins to the chloroplast (Maclean *et al.*, 2007) and the human codon-optimized HPV 6, 16, 34, 45, and 58 L1 genes cloned into pTRACTP were obtained from the Biopharming research unit (BRU) culture collection. These recombinant plasmids were confirmed by colony PCR using the vector specific primers listed in Table 1 (Section 2.2.3).

The other HPV L1 genes used in this study, HPV 18, 31, 33, 35 and 52, were obtained from GenScript in pUC57. All the genes contained 5' *Mlu*I and 3' *Xho*I restriction enzyme sites that allowed for subcloning into the pTRACTP plant expression vector. The L1 genes were excised from pUC57 using *Mlu*I/*Xho*I restriction enzymes and gel-purified after which they were directly ligated into pTRACTP that was linearised with the same restriction enzymes. The ligated constructs were transformed into competent *E. coli* DH5α cells and incubated overnight at 37°C. Multiple single colonies were analysed by colony PCR as described in Section 2.2.3, to screen for pTRACTP-L1 positive clones.

2.2.5 Transformation of *Agrobacterium*

The pTRACTP vector and pTRACTP-L1 plasmids for each HPV type was electroporated into electrocompetent *Agrobacterium* cells according to a method described by Shen and Forde (1989). Briefly, 100µl of competent *Agrobacterium* cells was mixed with 300ng of each recombinant pTRACTP-L1 HPV type in a 0.1cm

electroporation cuvette (Bio-Rad) and chilled on ice for 5 minutes. Cells were then electroporated at 200Ω, 1.8kV, and 25µF using a Gene Pulser Xcell™ (Bio-Rad) after which 900µl of LB medium was added. Cells were then allowed to recover by incubating them at 27°C for 2 hours with gentle agitation before being plated onto LB agar plates supplemented with the appropriate antibiotics and incubated for 2-3 days. Colony PCR with the pTRACTP specific primers was carried out as described in Section 2.2.3 to confirm successful transformation of *A. tumefaciens*. To further confirm the recombinant L1 plasmids, DNA isolated from positive *A. tumefaciens* constructs were back-transformed into *E. coli* DH5α cells. Plasmid DNA isolated from recombinant *E. coli* was digested with *Mlu*I and *Xho*I restriction enzymes.

2.2.6 *Agrobacterium*-mediated transient expression of *N. benthamiana* leaves

Previous research conducted in the BRU had focused on optimization of HPV L1 expression with the pTRACTP vector in *N. benthamiana* (Chabeda *et al.*, 2019, Maclean *et al.*, 2007). Therefore, the different HPV L1 proteins were expressed as follows: plants were infiltrated at optical density of 0.5 (OD_{600}=0.5) and biomass was harvested at 5 days post infiltration (dpi). The same infiltration conditions were used for the negative control, pTRACTP.

For large scale production of HPV VLPs, leaves of 6 weeks-old *N. benthamiana* (~40 plants per HPV type) were vacuum infiltrated with the recombinant *Agrobacterium* cultures at a final OD_{600} of 0.5. Briefly, 2ml of the recombinant *Agrobacterium* frozen glycerol stocks were revived by inoculating into 10ml LB medium supplemented with the relevant antibiotics and grown overnight with agitation. The following day, the 10ml pre-inoculums were inoculated into 50ml LB medium and grown overnight. The 50ml cultures were further scaled up the following day into a bigger flask containing 500ml LB supplemented with the relevant antibiotics (except rifampicin) and 20µM acetosyringone and grown overnight. Cultures were prepared for infiltration by diluting the 500ml overnight cultures to the required density of OD_{600} 0.5 in resuspension buffer (5mM MES, 10mM $MgCl_2.6H_20$, pH 5.6), 200µM acetosyringone was added to the diluted culture. To allow induction of the *vir* genes by acetosyringone, diluted cultures were incubated at room temperature for an hour prior to infiltration. Plants were vacuum infiltrated with OD 0.5 of Agrobacterium culture using -100kPa vacuum pressure before releasing it. Before and after infiltration plants were grown under the

following conditions: 22°C, 8 hours dark, and 16 hours of light. The pTRACTP negative control was treated and infiltrated in the same manner as all HPV constructs.

2.2.7 Extraction and purification of VLPs

Leaves were harvested 5 dpi and frozen at -80°C until protein extraction. For protein extraction, frozen leaves were homogenised with a T 25 digital Ultra-Turrax® (IKA® Works Inc) in 2x volumes of extraction buffer (1x High salt (0.5 M NaCl) Phosphate Buffered Saline, pH 7.4 [HSPBS]) (Maclean *et al.*, 2007), with cOmplete™, ethylenediaminetetraacetic acid (EDTA)-free Protease Inhibitor and incubated at 4°C with agitation for 2 hours. To remove large plant debris, homogenates were filtered through 4 layers of 22-24µm pore Miracloth™ (MilliporeSigma) and further clarified by centrifugation for 15 minutes at 15 317 x *g*, 15°C in an Avanti centrifuge (Beckman).

Clarified crude extracts were loaded onto 5ml 30% (w/v) sucrose cushion overlaid onto 1ml 50% sucrose cushion in 38ml Ulta-Clear™ ultracentrifuge tubes and centrifuged for 45 minutes at 174 587 x *g*, 15°C using a SW32Ti rotor (Beckman). All sucrose solutions were prepared in 1x HSPBS. After centrifugation, the 30% cushions were collected with a long needle and the sucrose removed by dialysing the samples overnight at 4°C in 1x HSPBS with agitation.

The following day, the dialysed samples were loaded onto discontinuous Optiprep™ (Sigma-Aldrich®) density gradients (5ml 27%, 4ml 33%, 2ml 39%, 1ml 50%) and centrifuged for 3 hours 30 minutes at 174 587 x *g*, 15°C using a SW32Ti rotor (Beckman). Optiprep™ is a sterile, endotoxin tested iodixanol-based gradient density medium, usually used for purification and isolation of macromolecules. Optiprep™ is obtained as a 60% iodixanol solution. To ensure that all the gradient steps contain the same PBS/NaCl concentration, a 50% Optiprep™ stock solution was made by diluting the Optiprep™ in 6x HSPBS. The 39%, 33% and 27% Optiprep™ steps were prepared from the 50% stock solution diluted with 1x HSPBS. The constant NaCl concentration maintained throughout the gradients ensures that the VLPs remain stable during purification. After centrifugation 1ml factions were collected from the bottom of the tubes by puncturing the lowermost part of the tube.

2.2.8 Protein analysis

All the HPV L1 proteins were detected on western blots, however HPV 52 L1 proteins were analysed on Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) since the rabbit-raised Gardasil® antiserum that was used to detect denatured HPV L1s in this study, unreliably detected HPV 52 L1s on western blot and there was no other antibody for detection of HPV 52 L1. The serum used in this study is not commercially available and was obtained by inoculating rabbits with Gardasil® by our lab. The serum has been tested and found to detect L1 of HPV 6, 16, 18, 31, 33, 34, 35, 45, and 58 (Alta van Zyl, personal communication).

Proteins were denatured by heating with 1x sample application buffer (SAB) (0,001% (w/v) bromothymol blue, 0.5M EDTA, 5% (w/v) SDS, 25% (v/v) glycerol) for 5 minutes at 95°C (Maniatis *et al.*, 1982, Sambrook *et al.*, 1989). The L1 proteins were resolved on 10% SDS-PAGE gels at 120V and either stained overnight at room temperature in Coomassie Blue stain (0.1% (w/v) brilliant blue G-250; 48% v/v methanol, 15% v/v glacial acetic acid) and then destained with destain solution (30% v/v methanol,10% v/v glacial acetic acid) overnight at room temperature. For western blots SDS-PAGE gels were transferred for 90 minutes at 15V onto nitrocellulose membranes with a semi-dry transblotter (transBlot®, Bio-Rad). Membranes were blocked in blocking buffer (1x PBS; 5% long life fat-free milk, 1% Tween20) at room temperature for 30 minutes with agitation. The membranes were probed with Gardasil® rabbit antiserum diluted in blocking buffer (1:2000 dilution) by incubating at 4°C for overnight with agitation. The blots were then washed 4 x 15 minutes in blocking buffer and incubated for 1 hour at 37°C with anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich®) diluted in blocking buffer (1:5000) with shaking. After incubation with the secondary antibody the membranes were washed 4 x 15 minutes with blocking buffer not containing milk. L1 proteins were detected after incubating the membranes with 5-bromo-4-chloro-3-indoxyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) phosphatase substrate (BCIP/NBT 1-component) (Whitehead Scientific) for 30 minutes.

2.2.9 Transmission electron microscopy

TEM was used to determine if the expressed L1 proteins fully assembled into VLPs. A Model 900 SmartSet Cold Stage controller (Electron Microscopy Sciences) was

used to glow discharge carbon-coated copper grids (mesh size 200) at 25mA for 30 seconds. Samples were trapped onto the grids by floating the grids, carbon side down, on the sample for 4 minutes. Thereafter, the grids were washed 4x with deionised water and negatively stained for 1 minute with 2% w/v uranyl acetate. Grids were air-dried and viewed with a FEI Tecnai 20 transmission electron microscope.

2.2.10 Vaccine candidates

2.2.10.1 Detection of selected vaccine candidates with monoclonal antibodies

As described in Section 2.1, HPV 35, 52 and 58 were selected for the immunogenicity study (Chapter 3). Therefore, conformation and structural integrity of HPV plant-produced VLP vaccine candidates were further analysed by carrying out dot blots, using type-specific monoclonal antibodies (MAbs) from Dr Neil Christensen (Penn State Cancer Institute) (Table 2.2) that detect intact proteins. These MAbs bind to the conformational epitopes on the surface of non-denatured VLPs and/or capsomeres. Therefore detection of L1 by these types partially suggest correct folding and assembly of particles that display conformational epitopes (Christensen *et al.*, 1996a). Purified empty vector pTRACTP was used as negative control. Additionally, each HPV type was probed with all 3 type-specific MAbs, to ensure there was no cross-contamination during sample preparation.

A volume of 2µl of purified plant-produced VLPs and negative control pTRACTP was added on the nitrocellulose membrane and left to air-dry. Membranes were then blocked with blocking buffer for 30 minutes with agitation. Membranes were probed with type-specific primary MAbs (table 2.2) diluted in blocking buffer (1:1000) and incubated at 4°C overnight with agitation. The blots were washed 4 x 15 minutes in blocking buffer and incubated for an hour with agitation at 37°C with anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich®) diluted in blocking buffer (1:5000). Afterward, the membranes were washed 4 x 15 minutes with blocking buffer without milk. Protein was detected by incubating the membranes with BCIP/NBT 1-component substrate for 10 minutes.

Table 2.2: Monoclonal antibodies used to detect purified plant-produced HPV vaccine candidates

HPV types	MAbs
HPV 35	H35Q8
HPV 52	H52D11
HPV 58	H58 J6.3

2.2.10.2 Protein quantification of selected vaccine candidates

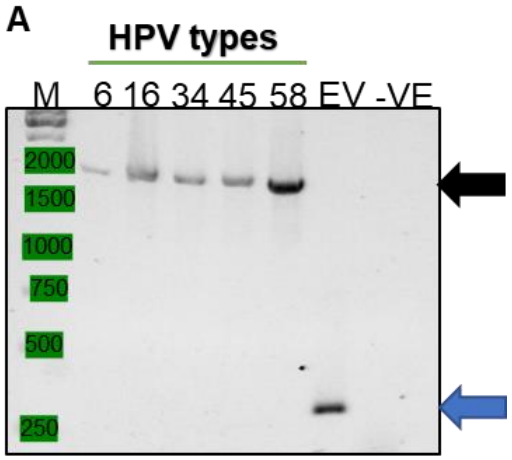
Gel densitometry was used to quantify plant-produced HPV vaccine candidates. Bovine serum albumin (BSA, (Sigma-Aldrich®)) was used as protein standard. BSA (1mg/mL) was diluted 2-fold in PBS to generate a standard curve. BSA standards and 25µl of samples containing VLPs were mixed with 1x SAB and denatured at 95°C for 5 minutes. A volume of 25µl of the standards and the samples were loaded onto a 10% SDS-PAGE gel. The gel was Coomassie-stained and destained as described in Section 2.2.8. Quantification of the protein bands was carried out with StudioTM Lite version 5.2 software (LI-COR®).

After quantification, the concentration needed for mice vaccinations was calculated for each type. Samples were pooled and then aliquoted into volumes enough for each injection. Samples were tested for endotoxin using ToxinSensor™ chromogenic LAL endotoxin assay kit (GenScript) as per manufacturer's instructions. Furthermore, samples were grown overnight on LB agar at 37°C without antibiotics and observed for any viable bacterial growth before they were stored at -80°C until animal studies. The plant purified negative control was treated the same way.

2.3 Results

2.3.1 Recombinant HPV plasmids

A. tumefaciens transformed with human codon-optimized HPV 6, 16, 34, 45, and 58 L1 genes already cloned into the pTRACTP plant expression vector were provided by our lab and confirmed by colony PCR (Figure 2.2A). L1 positive clones are indicated by the presence of bands at ~1500 base pairs (bp) (black arrow, Figure 2.2A). As expected, the empty vector yielded a product at ~300 bp (blue arrow), and no band was observed in the PCR negative control (Figure 2.2A). Meanwhile, human codon-optimized HPV 18, 31, 33, 35 and 52 L1 genes were excised from pUC57 with *Mlu*I/*Xho*I restriction digestion and sub-cloned into pTRACTP. Recombinant pTRACTP-HPV L1 plasmids were transformed into competent *E. coli* DH5α cells and confirmed by colony PCR (Figure 2.2B) before electroporation into *A. tumefaciens* GV3101::pMP90RK. For *E. coli* and *Agrobacterium*, all selected colonies tested positive for the presence of L1, as indicated by the expected band size of ~1500bp (black arrow, Figure 2.2B and C, respectively). Furthermore, no band was observed in the no template PCR negative control for both *E. coli* DH5α and *Agrobacterium* transformation (Figure 2.2B and C, respectively). Restriction enzyme digests were carried out to further confirm the recombinant clones (data not shown).



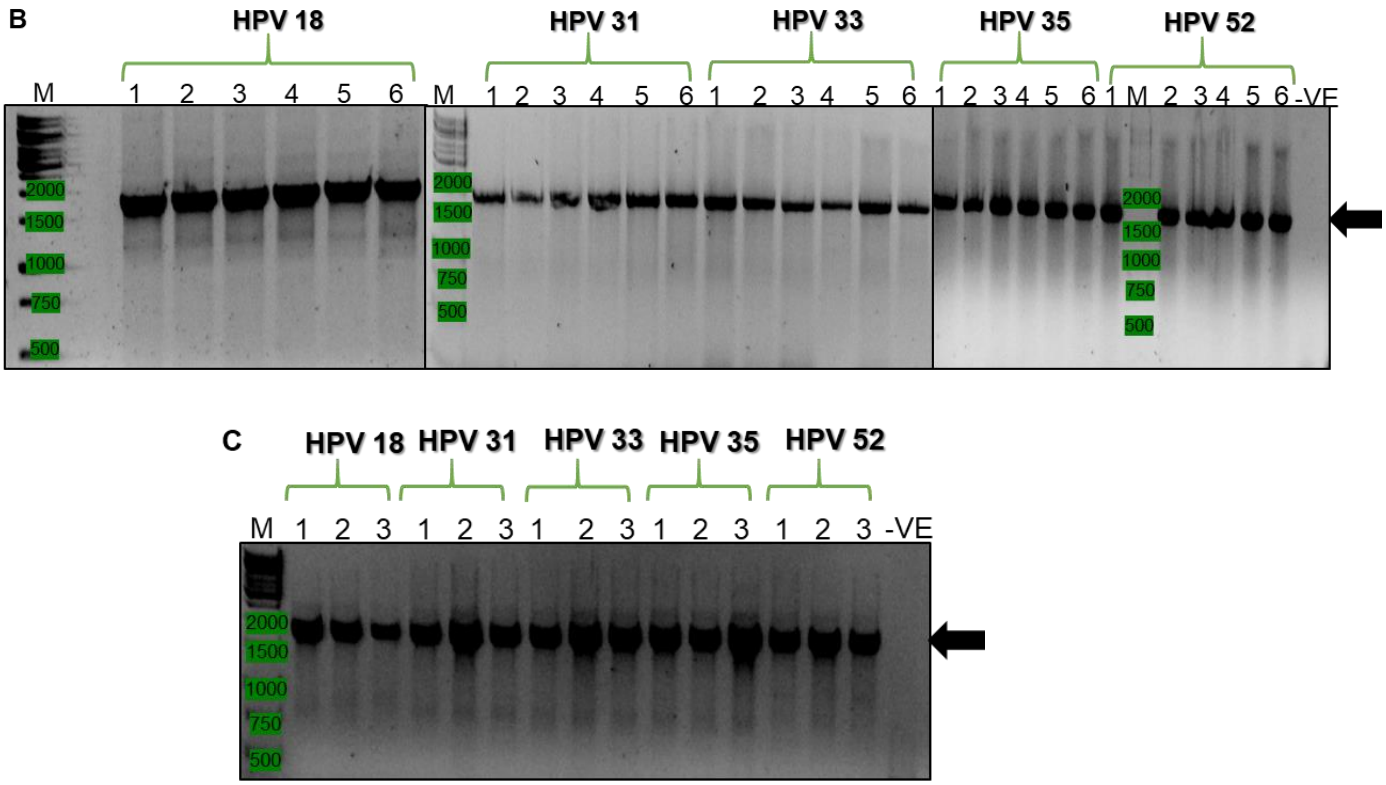


Figure 2.2: Colony PRC of recombinant pTRACTP-HPV L1 genes: A) confirmation of HPV L1 clones in *Agrobacterium* obtained from BRU culture collection; B) Colony PCR confirming successful *E. coli* transformation and *Agrobacterium* transformation (C). The presence of PCR fragments at ~1500 bp (black arrows) indicate L1 positive clones. As expected, the empty vector yielded approx. 300 bp product (A, blue arrow) and no bands were observed in the PCR negative controls. Labels: -VE: no DNA template PCR control; EV: empty pTRACTP vector; Numbers 1-6 and 1-3 - selected colonies of each HPV type; M: DNA ladder with sizes indicated in base pairs.

2.3.2 Large scale expression of HPV L1 proteins in *N. benthamiana*

The aim was to evaluate plant-based transient expression as a tool for making L1 VLPs of HPV 6, 16, 18, 31, 33, 34, 35, 45, 52 and 58 that are potentially suitable for making affordable prophylactic HPV vaccines, especially for use in developing countries. To date, plant-based studies have been focusing mainly on the development of HPV 16 L1 and L1/L2 VLPs in plants. This gave rise to this study, which investigated the expression of VLPs for other oncogenic HPVs in plants.

Expression of HPV L1 genes in *N. benthamiana,* was previously optimized by our lab (Chabeda *et al.*, 2019, Maclean *et al.*, 2007). Large scale expression studies were

performed by vacuum infiltration with recombinant *Agrobacterium* culture at a final OD_{600} of 0.5. At 5 dpi all plants looked relatively healthy with the development of slight chlorosis in plants infiltrated with HPV L1 as compared to empty vector, no tissue necrosis was observed. Figure 2.3 shows representative phenotypes of leaves infiltrated with selected vaccine candidates (A-B) and empty vector (D). Leaves of plants infiltrated with HPV L1 had a slight change in colour to pale yellow (Figure 2.3A-C) when compared to those infiltrated with empty vector (Figure 2.3D). Leaves of plants infiltrated with other 7 HPV types had similar phenotypes as that observed for vaccine candidates. Leaves were harvested 5 dpi and frozen at -80°C until extraction and purification.

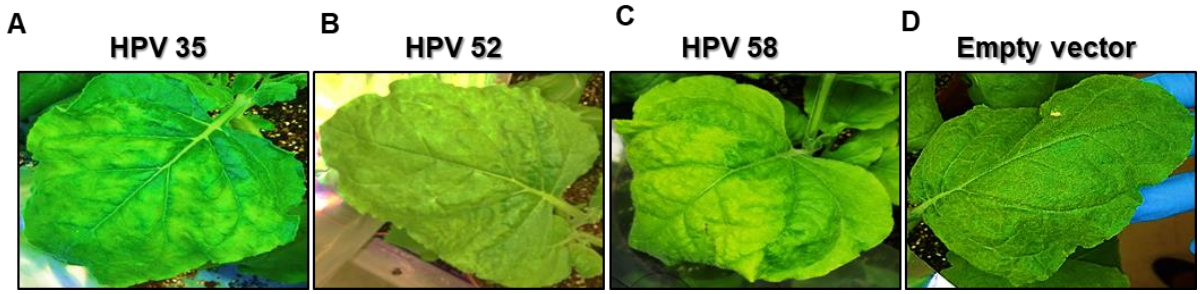


Figure 2.3: Phenotype of infiltrated plant leaves: A-C) leaves of HPV 35, 52 and 58, respectively; D) Empty vector. All plants were infiltrated at OD_{600} 0.5 and harvested 5 dpi.

2.3.3 Purification and analysis of HPV L1 proteins

To assess if L1s were expressed in tobacco plants, leaves were extracted and purified as described in Section 2.2.7. Expression of L1s were determined by their sedimentation in Optiprep™ step-gradients, detection by rabbit-raised Gardasil® antiserum on western blots and Coomassie-blue staining in the case of HPV 52.

Prior to purification of the L1 VLPs on Optiprep™ step-gradients, crude L1 plant extracts were concentrated in 30% sucrose cushions, Section 2.2.7. The 30% sucrose cushioned samples of all HPV types appeared green after centrifugation (yellow arrow, Figure 2.4A and B). The 30% sucrose fractions were collected from the tubes and dialysed overnight. The dialysate was further purified on discontinuous Optiprep™ gradients. After centrifugation a green band at or above the 27% step was observed (green arrow, Figure 2.4C and D), potentially indicating that co-extracted plant proteins are contained at the top of the gradient. A light brown band was observed in the 33%

and 39% Optiprep™ fractions (yellow arrow, Figure 2.4C and D), where assembled HPV particles were expected to occur. There was no pellet observed after ultracentrifugation. A volume of 1ml fractions were collected from the bottom of the tubes and analysed on western blots and Coomassie-stained gels in the case of HPV 52.

Initially it was attempted to concentrate plant-purified fraction on a second, smaller step gradient by banding the VLPs onto the 50% Optiprep™ fraction according to the method described by Chabeda *et al.* (2019). This however did not yield consistent results, therefore Optiprep™ steps making up the discontinuous gradients were halved in volume and finally 500 µl fractions were collected from the tubes.

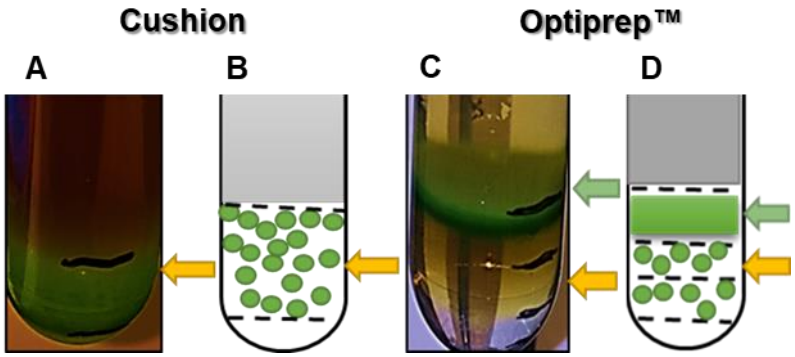
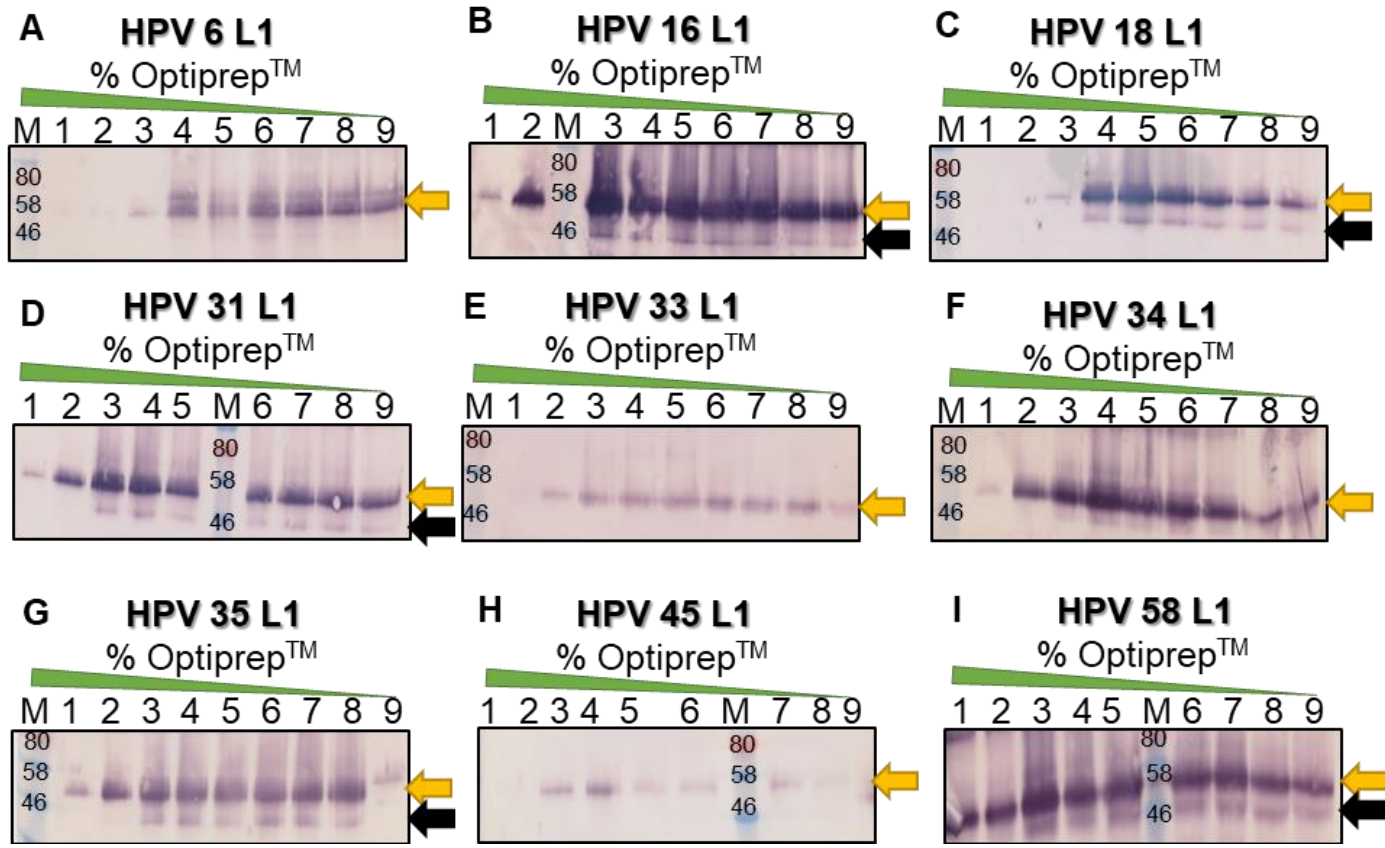


Figure 2.4: Purification of plant produced HPV VLPs: Photos and schematic representations of, A-B) sucrose cushion (30% and 50 %); C-D) Optiprep™ gradient (50%, 39%, 33% and 27%). Labels: yellow arrows-VLPs; green arrows-native plant proteins.

Western blot analysis with Gardasil® antiserum of fractions collected after centrifugation showed the presence of bands at ~56 kDa from fraction 2 onward, indicating the presence of HPV L1 after purification (yellow arrows, Figure 2.5A-J). Except in the case of HPV 16, 31, 34, 35 and 58 L1 was detected from fraction 1 onward. This possibly indicated the co-purification of VLPs and/or capsomeres or aggregates of L1 of dissimilar densities. For HPV 16, 18, 31, 35 and 58 a second band was detected at ~46 kDa, indicating potential cleavage products of L1 (black arrows, Figure 2.5B-D, G and I). By comparing the intensity of the bands on individual western blots, it is evident that lower expression levels of HPV 33 and HPV 45 L1 was obtained compared to the other HPV types. However, fraction 4 of all HPV types, except for HPV 52 were all analysed on one blot, which indicated the presence of L1 at ~56 kDa

with similar band intensity for all types (Figure 2.5J). Furthermore, the blot indicated that HPV 6, 16, 18, and 45 L1s run higher on western than other types.

HPV 52 was analysed on Coomassie-stained SDS-PAGE only, because there was no antibody to reliably detect HPV 52 L1 on western blot. The Coomassie-stained gel indicated the presence of HPV 52 L1 at ~56 kDa from fraction 2 onwards (Figure 2.5K). The empty vector (pTRACTP) negative control was analysed on a western blot and Coomassie-stained gel, as expected no L1 bands were observed on the western blot (Figure 2.5L). Two bands ~59 and 55 kDa were observed in fraction 9 of the Coomassie-stained gel, possibly indicating the presence of native plant proteins, such as RuBisCo, in less dense gradient fractions (Figure 2.5M).



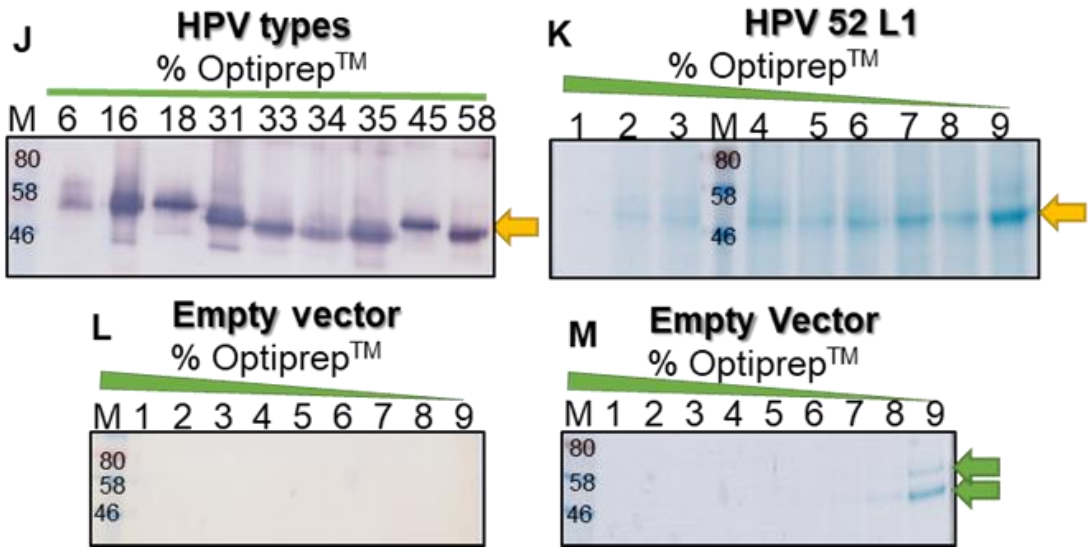


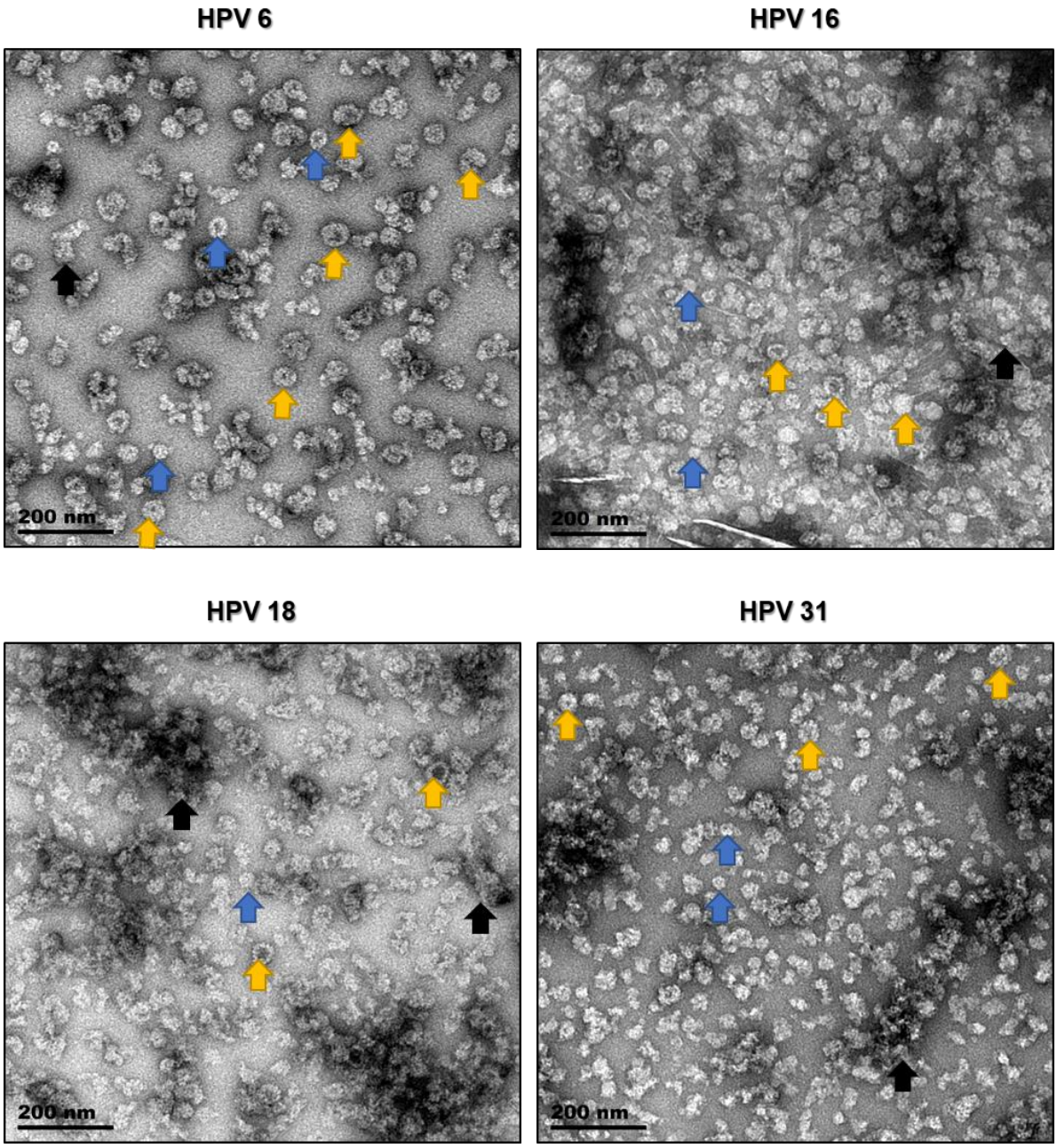
Figure 2.5: Analysis of plant produced HPV VLPs: A-I) individual HPVs western blots; J) western blot of all HPV types; K) Coomassie-stained gel for HPV 52; L-M) western blot and Coomassie-stained gel for the empty vector, respectively. Purified HPV L1 proteins (~56 kDa) were probed with anti-Gardasil® (1:2000 dilution) and anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (1:5000). Labels: M: pre-stained protein standard (kDa); yellow arrows: HPV L1 protein (~56 kDa); black arrows: potential L1 cleavage products (~46 kDa); green arrows: native plant proteins.

2.3.4 Transmission electron microscopy analysis

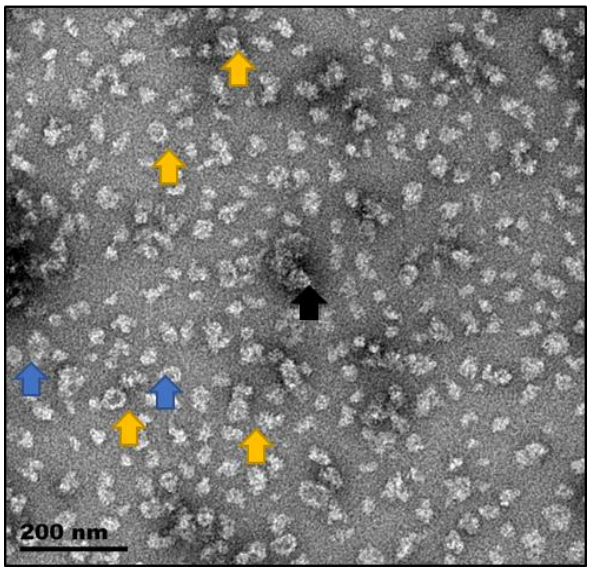
After successful expression of HPV L1s in *N. benthamiana*, the next step was to assess whether the L1 proteins successfully assembled into VLPs. This was achieved by trapping samples onto carbon-coated copper grids and viewed under TEM. TEM analysis showed that VLPs were mostly observed in 33 and 39% Optiprep™ (fractions 3-6). Figure 2.6 shows the TEM representative of fractions which had better looking VLPs, fraction 3 for HPV 16, 34, 35 and 45; fraction 4 for HPV 6, 52 and 58; and fraction 5 for HPV 18, 31 and 33. For all the HPV types expressed, TEM analysis showed fully assembled spherical VLPs of 40-60nm diameter (yellow arrows), small VLPs and/or capsomeres of 25-39nm diameter (blue arrows) and L1 aggregates (black arrows) (Figure 2.6). HPV L1 has been shown to assemble into particles of different sizes, varying from 25-60nm (Chabeda *et al.*, 2019, Pineo *et al.*, 2013, Kim *et al.*, 2010, Maclean *et al.*, 2007, Biemelt *et al.*, 2003).

By comparing VLPs per field of view HPV 6, 16, 35, 45, 52 and 58 appeared to be populated by fully assembled VLPs (40-60nm) as compared to HPV 18, 31, 33, and

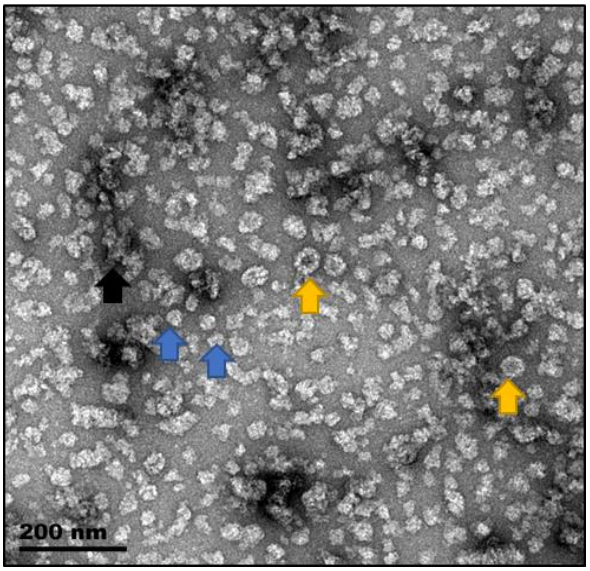
34 which were mostly populated by capsomeres and aggregates. Furthermore, HV 52 and 58 made particles with better structural integrity than other types. The population of fully assembled particles decreased across the fractions, less dense gradient fractions (F7-9) were mostly populated by L1 aggregates. No structures resembling VLPs and/or capsomeres were observed in the negative control, structures seen in the negative control is probably plant aggregates (venetian red arrow, Figure 2.6).



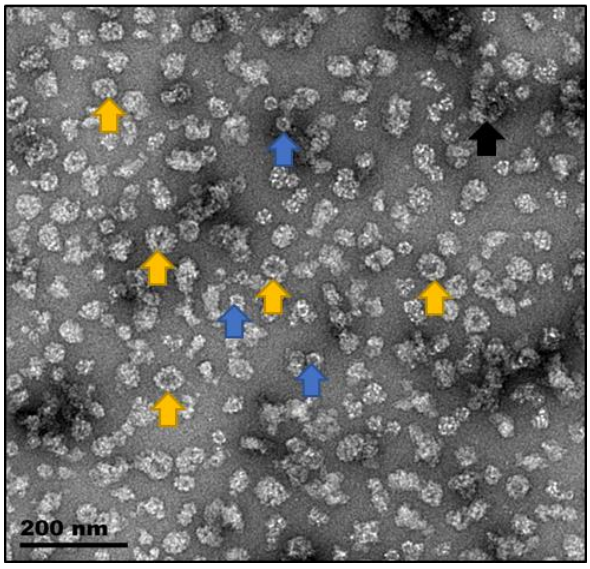
HPV 33



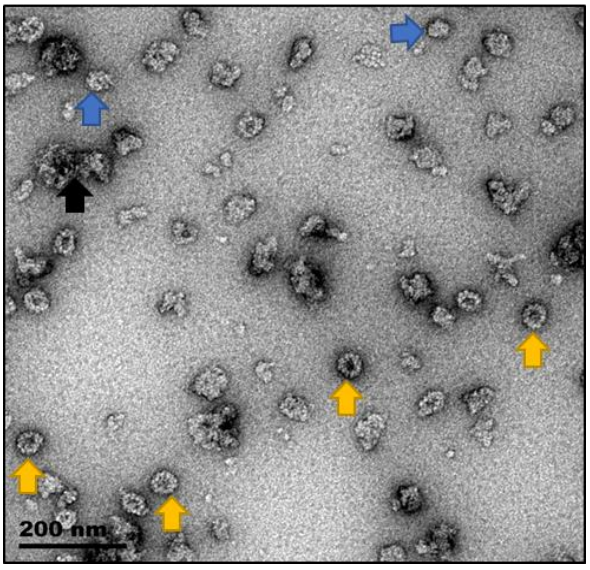
HPV 34



HPV 35



HPV 45



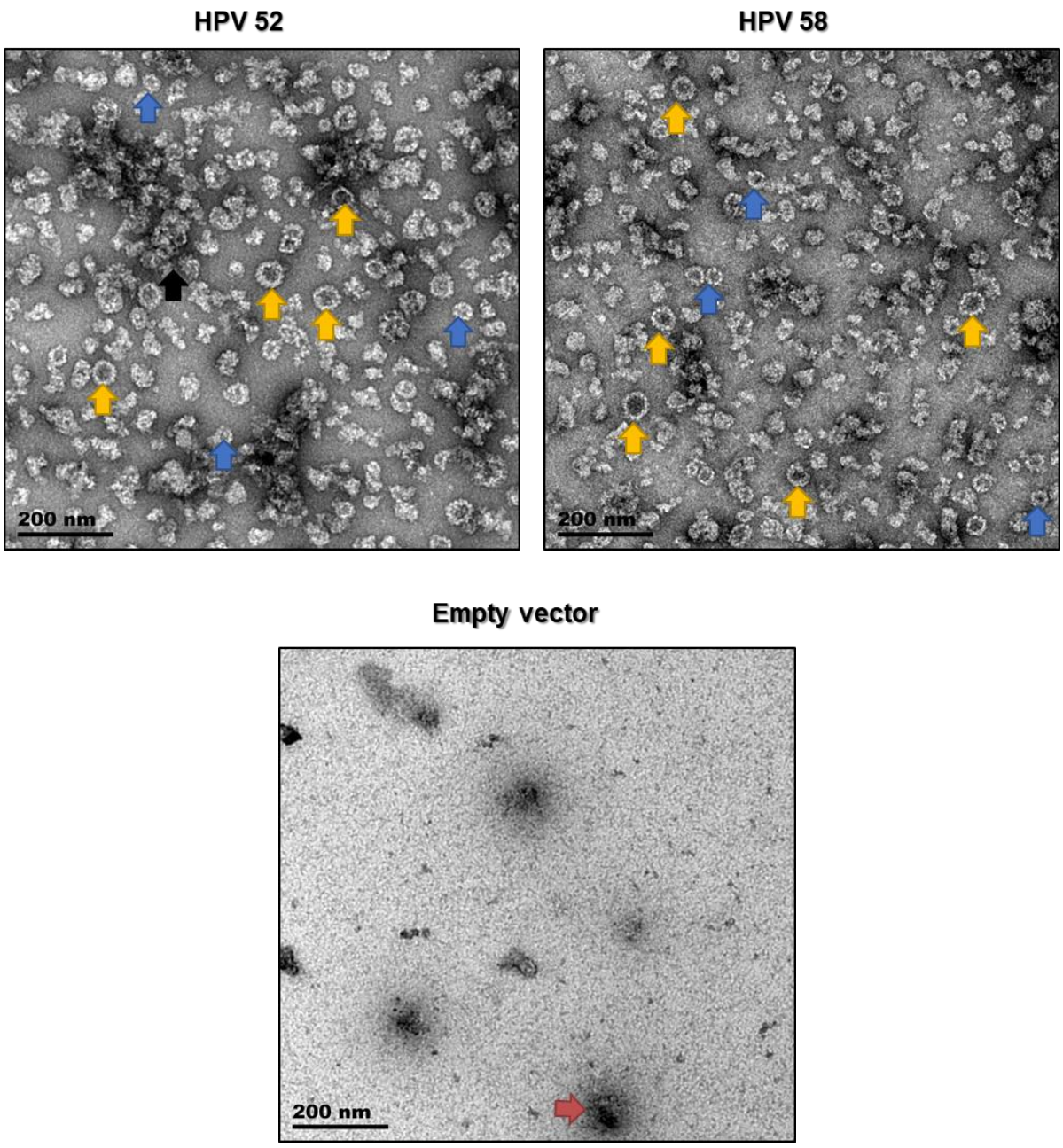


Figure 2.6: Electron micrographs of purified plant-produced L1 antigens: Samples were purified on Optiprep™ density gradients and viewed under EM at the magnification of 40 000 X. Bars are 200nm in size. Labels: yellow arrows: VLPs; blue arrows: small VLPs and/or capsomeres; black arrows: L1 aggregates; venetian red arrow: native plant aggregates.

2.3.5 Vaccine candidates

2.3.5.1 Detection of selected vaccine candidates with monoclonal antibodies

After showing that L1 successfully assembled into VLPs, I wished to determine whether the observed VLPs can induce an immune response in an animal model. However as explained in Section 2.1, only HPV 35, 52 and 58 were selected for the animal study. VLPs/capsomeres of vaccine candidates were tested to determine whether they displayed conformational epitopes, which usually results in high titres of neutralizing antibodies (Kirnbauer *et al.*, 1992).

The structural integrity and conformation of VLPs/capsomeres for selected vaccine candidates were tested by carrying out dot blots using type-specific monoclonal antibodies (Section 2.2.10.1, Table 2.2) which bind to the conformational epitopes on the surface of undenatured L1 VLPs and/or capsomeres (Christensen *et al.*, 1996a). To ensure there was no cross-contamination during sample preparation, each HPV type was probed with all three type-specific MAbs. As expected, type specific monoclonal antibodies detected corresponding HPV L1 proteins (Figure 2.7), indicating that samples were not cross-contaminated. However, H35Q8 and H52D11 antibodies weakly reacted with HPV 58 and HPV 35 L1, respectively (Figure 2.7A and B). This suggested potential cross-reactivity due to shared conformational epitopes between these types. Detection of HPV L1s by these antibodies confirmed the presence of assembled particles displaying surface conformational epitopes in the samples. As expected, the antibodies did not bind to the purified negative control (empty vector) samples (Figure 2.7).

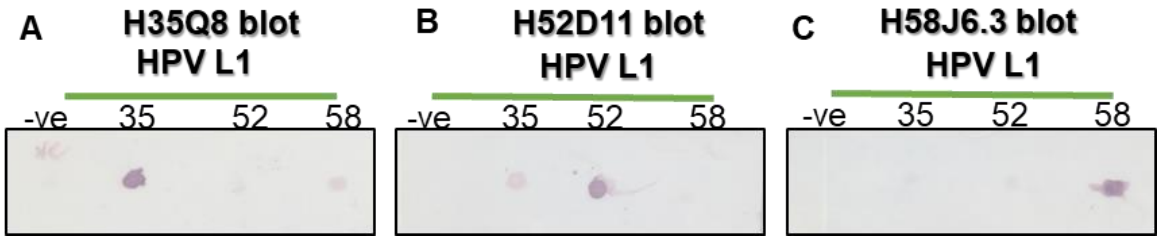


Figure 2.7: Dot blots of vaccine antigens: L1 proteins were detected using type specific monoclonal antibodies: HPV 35: H35Q8; HPV 52: H52D11; HPV 58: H58J6.3. All the antibodies were used at a dilution of 1:1000, followed by probing with anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:5000). Labels: -ve: negative control (empty vector).

2.3.5.2 Quantification of vaccine candidates

Selected vaccine candidates were quantified to determine the amount of VLPs and/or capsomeres present in the samples. Efforts to concentrate VLPs did not yield consistent results, therefore discontinuous gradients were halved in volume and 500µl fractions were collected. Fractions 3-5 (33% and 39% Optiprep^{TM}) had the best looking VLPs and/or capsomeres for the selected vaccine candidates. Therefore, they were each quantified using gel densitometry, with BSA as protein standard (results not shown) to determine the approximate VLPs/capsomeres in each fraction.

Total VLP and/or capsomere yields per kilogram (kg) of fresh weight biomass of at least ~7.28mg/kg, 5.44mg/kg and 4.36mg/kg were obtained from fractions 3-5 for HPV 35, 52 and 58 respectively. After the concentration needed for mice vaccinations was calculated for each type, samples were pooled and tested for bacterial endotoxin level. Approximately 14EU/ml was estimated in the empty vector sample, whereas VLPs sample had a slightly higher level of 50EU/ml. This amounted to a total of ~0.84EU and 3EU per injection of empty vector and VLPs sample respectively. Furthermore, samples were grown overnight on LB agar plates to ensure no viable bacterial growth present, which might negatively affect the immune response and cause harm to the mice. No bacterial colonies were observed, and samples were stored at -80°C until animal study.

2.4 Discussion

Commercially available prophylactic HPV vaccines are effective at preventing HPV infections (McClymont *et al.*, 2019, Wilkin *et al.*, 2018, Lin *et al.*, 2010, Moodley *et al.*, 2006). However, the cost associated with the commercially available HPV vaccines is preventing their use in developing countries especially Africa, where the highest burden of cervical cancer is reported (Liu *et al.*, 2005, Taira *et al.*, 2004). A multivalent vaccine targeting all HPVs that are important and more dominant in Africa may alleviate the high cervical burden reported in this region. Plants have been utilized with the hope of producing affordable vaccines and might overcome the disease burden in developing countries (Rybicki, 2010, 2009, Liu *et al.*, 2005). Plants produced vaccines can potentially reduce the estimated vaccine cost with up to 31% at the production level (Rybicki, 2009). For these reasons, efforts to develop affordable HPV vaccine candidates in plants are under way.

HPV 16 L1 and/or L1/L2 chimaeric VLPs have successfully been transiently expressed in plants and have been shown to elicit immune response in animal models (Chabeda *et al.*, 2019, Pineo *et al.*, 2013, Fernández-San Millán *et al.*, 2008, Maclean *et al.*, 2007, Varsani *et al.*, 2006). In this study, transient expression of human codon-optimized HPV L1 genes in *N. bethamiana* was utilized to develop HPV L1 VLPs, that will potentially be used for making affordable multivalent prophylactic HPV vaccines, for use in developing countries. Five days post infiltration, plants looked healthy with minimum if any, chlorosis observed (Figure 2.3). These findings were similar to research done by Chabeda (2017), where expression of HPV 16 L1 and L1:L2 chimaeras with the same vector used in this study (pTRACTP) resulted in increased protein expression with minimal development of chlorosis and necrosis.

Purity and efficaciousness are essential when preparing VLPs for vaccination purposes (Vicente *et al.*, 2011). Therefore, robust downstream processing of VLP-based vaccines is crucial. Density gradient ultracentrifugation is regularly used for the purification of VLPs from several expression systems at the laboratory level.HPV VLPs have been purified by ultracentrifugation in other studies (Chabeda *et al.*, 2019, Minkner *et al.*, 2018, Jiang *et al.*, 2011, Park *et al.*, 2008, Maclean *et al.*, 2007, Buck *et al.*, 2005a). In the present study, plant-made VLPs were purified in two steps, firstly by concentrating the VLPs present in the crude plant extract in a 30% sucrose cushion.

In the second step the dialysed 30% sucrose fractions were loaded onto Optiprep™ step gradients to enable for the purification of HPV VLPs based on their buoyant density, isopycnic centrifugation (van Zyl and Hitzeroth, 2016).

After ultracentrifugation, successful separation of plant proteins from HPV L1 VLPs were obtained in that plant proteins were detected in fractions 8 and 9 (27% Optiprep™, Figure 2.5M), this was also evidenced by the presence of a green band at or above the 27% step in the Optiprep™ gradient (Figure 2.4C and D). These results are consistent with previous results obtained in our lab. In Chabeda *et al.* (2019), a low pH buffer was used for purification of chimaeric VLPs, and although the low pH buffer is effective in reducing the amount of host cell proteins co-purified with L1, it also results in significant L1 losses compared to purification of L1 VLPs in neutral pH buffer. These indicate that there is still much to be done to improve purification techniques of plant-made HPV VLPs.

The presence of bands ~56 kDa on western blots and Coomassie-stained gel in the case of HPV 52 (Figure 2.5), indicated successful expression and purification of the L1 capsid protein in *N. bethamiana*. These results are consistent with other studies (Chabeda *et al.*, 2019, Pineo *et al.*, 2013, Fernández-San Millán *et al.*, 2008, Maclean *et al.*, 2007, Biemelt *et al.*, 2003). Furthermore, potential cleavage products of L1 (bands ~46 kDa) were observed on westerns with some HPV types (black arrows, Figure 2.5). Protein degradations within tobacco plants have been reported in other studies (Chabeda *et al.*, 2019, Veerapen *et al.*, 2018, Fernández-San Millán *et al.*, 2008, Sharp and Doran, 2001, De Neve *et al.*, 1993). Protein degradation may be explained by thousands of proteases such as: cysteine proteases present in plants. In plants, proteins are degraded either inside the cells by intracellular proteases or outside by extracellular proteases (Doran, 2006, Sharp and Doran, 2001). Among other proteases, the genome of tobacco is known to code for a minimum of 60 putative cysteine proteinases (CysPs), which might be involved in protein degradation (Duwadi *et al.*, 2015, Hao *et al.*, 2006).

TEM analysis showed fully assembled VLPs measuring 40-60nm in diameter as well as small VLPs and/or capsomeres measuring 25-39nm (Figure 2.6). L1 aggregates were also observed under TEM. HPV L1 has been shown to assemble into particles of different sizes, varying from 25-60nm in different expression systems, including

plants (Chabeda *et al.*, 2019, Pineo *et al.*, 2013, Kim *et al.*, 2010, Maclean *et al.*, 2007, Kohl *et al.*, 2007, Biemelt *et al.*, 2003). Full sized (40-60nm) and small (25-39nm) plant-produced HPV 16 L1 VLPs have been observed in the chloroplast (Chabeda *et al.*, 2019, Pineo *et al.*, 2013, Maclean *et al.*, 2007, Biemelt *et al.*, 2003). The presence of L1 aggregates after purification was not surprising as it appears that recombinantly produced HPV L1 is prone to aberrant assembly, as the presence of aggregates have been reported on in other research (Chabeda *et al.*, 2019, Maclean *et al.*, 2007, Kohl *et al.*, 2007, Biemelt *et al.*, 2003). However, formation of L1 aggregates may not be unique to plants, HPV VLPs in other expression systems - yeasts and insect cells are made by *in vitro* disassembling/reassembling of purified L1 to get a homogenous population of VLPs, suggesting the formation of aggregates by these systems (Zhao *et al.*, 2014).. Therefore, plant transient-based expression system can be used as a tool for making cost-effective HPV L1 VLPs that can potentially be explored to make prophylactic HPV vaccines.

After successful expression of the 10 HPV L1 VLPs in tobacco plants, the second step was to test whether these VLPs are appropriate for the development of affordable HPV prophylactic vaccines. However, to reduce the number of animals used, only 3 plant-produced HPVs (35, 52 and 58) were selected for mice studies (Chapter 3). These types have been selected mainly for their importance in Africa, especially Sub-Saharan Africa as described in section 2.1. HPV L1 VLPs selected for mice immunization experiments were then further analysed by probing with type-specific conformational antibodies on dot blots (Figure 2.6). Detection of the proteins by these antibodies confirmed that even though a homogenous population of VLPs was not obtained after purification, conformational epitopes were displayed on the VLPs and/or capsomeres present in the purified samples. This suggested the potential of these VLPs to induce immune response, therefore preventing HPV infection.

Selected HVP vaccine candidates were quantified using gel densitometry. Prior to storage of the vaccine candidates at -80 °C, 2 bands at 56 and 46 kDa were observed on western blots for HPV 35 and HPV 58 (Figure 2.5G and I). The lower molecular weight band observed on blots could have been due to cleavage of L1 proteins by host cell proteases such as cysteine proteases. In the case of HPV 52 no lower molecular weight band was observed immediately after extraction and purification however, after freezing and thawing, the lower molecular band was observed (results not shown).

This indicated degradation of L1 protein during storage, which was also reported on in Pineo *et al.* (2013). The expected L1 band at 56 kDa and the lower molecular weight band at 46 kDa were quantified to obtain the total amount of L1 VLPs and/or capsomeres present in collected fractions with best looking VLPs based on TEM micrographs.

In this study, expression of human codon-optimized HPV L1 genes resulted in accumulation of HPV L1 VLPs in the chloroplast. Efforts to concentrate HPV L1 did not yield consistent results, therefore the total L1 yields were not determined in this study. However, total VLP yields per kg of fresh weight biomass of at least ~7.28mg/kg, 5.44mg/kg and 4.36mg/kg were obtained from fractions 3-5 for HPV 35, 52 and 58 respectively. Total L1 yield of 533mg/kg and 142mg/kg was reported by other researchers when codon-optimized HPV 16 L1 genes were transiently expressed in tobacco using chloroplast-targeted vectors (Chabeda *et al.*, 2019, Maclean *et al.*, 2007). Fernández-San Millán *et al.* (2008) reported the total L1 of 3000mg/kg in tobacco plants. The higher expression level was achieved using HPV 16 L1 fused to the 5′-untranslated region (5′-UTR) of the *psbA* gene of the pAF vector to transform tobacco chloroplast. The authors noted that 5'-UTR might have caused high translation rate, which might explain the higher expression level achieved compared to other studies.

Early efforts to produce affordable HPV vaccines in plants included the expression of humanized codon-optimized HPV 16 L1 gene and plant-codon optimized HPV 11 L1 gene in transgenic tobacco and potato (Biemelt *et al.*, 2003, Warzecha *et al.*, 2003). Although HPV L1 genes successfully assembled into immunogenic VLPs, the expression level was very low. Varsani *et al.* (2006) transiently expressed native HPV 16 L1 genes in *N. benthamiana*, however the expression level was still low.

Maclean *et al.* (2007) compared plant and human codon optimized HPV L1 genes. The study showed that the expression of human codon optimized HPV L1 genes ensured optimal usage of transfer ribonucleic acid (tRNA) and translation by plant cellular machinery and therefore resulted in high protein expression levels. Whereas the expression of plant codon-optimized L1 gene did not yield detectable L1 protein (<1mg/kg of plant biomass). Furthermore, targeting the protein to different plant cell compartments influenced protein expression. Maclean *et al.* (2007) showed that when

the proteins were targeted to the chloroplast a yield of 533mg/kg was achieved, whereas when they were targeted to the cytoplasm a lower yield of 379mg/kg was observed.

Increased accumulation of recombinantly expressed HPV L1 proteins in the chloroplast has also been reported by other researchers (Chabeda *et al.*, 2019, Zahin *et al.*, 2016, Pineo *et al.*, 2013, Fernández-San Millán *et al.*, 2008). Additionally, expression of Type I interferons -α2b, cholera toxin B (CTB) fusion proteins, Anthrax protective antigen, and CTB subunit gene in plants resulted in high level of protein expression in the chloroplast (Arlen *et al.*, 2007, Limaye *et al.*, 2006, Watson *et al.*, 2004, Daniell *et al.*, 2001). Apart from high translation rate by 5′-UTR in the vector as reported by Fernández-San Millán *et al.* (2008), the increased level of protein accumulated in this organelle may be explained by diverse protein hydrolysis, low level of cell toxicity, stable messenger RNA (hence stable L1 protein in this case), and low level of proteases in this compartment (Maclean *et al.*, 2007). Consequently, high expression levels of HPV L1s recently achieved when targeting the chloroplast illustrate that tobacco plants are a promising system to produce affordable HPV vaccines for use in resource-poor countries.

In conclusion, the major L1 capsid protein of 10 HPV types were successfully expressed in *N. bethamiana* and purified by density gradient ultracentrifugation. TEM analysis of the purified L1 proteins showed the successful assembly of VLPs for all 10 HPV L1 types. Three HPV types (35, 52 and 58) were selected and prepared for mice immunization studies (chapter 3). The study has demonstrated that transient production of HPV L1 VLPs in tobacco plants, can potentially be used to develop affordable HPV prophylactic vaccines. This is the first study that expressed 10 HPV L1s in plants, marking a step towards the development of cheaper multivalent HPV vaccine, for use in developing countries.

3 Chapter 3: Immunogenicity of plant-made HPV VLPs in mice

3.1 Introduction

Vaccination is the most effective approach in preventing viral diseases. It results specifically in a humoral immune response with neutralizing antibodies, that enable the prevention of infections (Schiller and Lowy, 2018). HPV VLPs are structurally identical to native virions and most importantly they display high density of conformational and/or linear neutralizing epitopes (Grgacic and Anderson, 2006). HPV VLPs are effective at preventing infections and is the foundation onto which commercially available vaccines have been developed.

Three prophylactic VLPs-based HPV vaccines are commercially available- Gardasil®, Cervarix™ and Gardasil9®. Gardasil® targets HPV 6, 11, 16 and 18, whereas Cervarix™ protects against HPV 16 and 18 infections (Schiller *et al.*, 2008). Gardasil9® targets a wide spectrum of HPV types (HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58) (Petrosky *et al.*, 2015). These vaccines are all formulated in aluminum-based adjuvants and studies have demonstrated that they are highly effective and safe. Significant reduction in high-grade cervical abnormalities and genital warts have been reported in Australia since the government introduced national vaccination programme with Gardasil® for women aged 12–26 years (Brotherton *et al.*, 2011). While, vaccination of women aged 16-24 years with Cervarix® reduced the prevalence of HPV 16 and 18 from 19.1% to 6.5 % in England (Mesher *et al.*, 2013).

Studies focusing on the safety of Gardasil® and Cervarix™ vaccines, concluded that, they are safe and well-tolerated (Clark *et al.*, 2013, Roteli-Martins *et al.*, 2012, Gasparini *et al.*, 2011, Block *et al.*, 2010, Didierlaurent *et al.*, 2009). Gardasil9® has been shown to prevent persistent infections and vaginal, vulvar and cervical diseases caused by the HPV types included in the vaccines (Joura *et al.*, 2015). Although the current commercially available HPV vaccines are effective at preventing HPV infections, the high costs associated with these vaccines prevents vaccination programmes in developing countries, where they are most needed (McKee *et al.*, 2015, Biemelt *et al.*, 2003). For example, Africa has the highest burden of cervical cancer (Ginsburg *et al.*, 2017), yet only 1-2% of girls aged 10-20 years get vaccinated in this region with only 7 out of 54 countries having national HPV vaccination

programmes (Bruni *et al.*, 2016, Chido-Amajuoyi *et al.*, 2019). Therefore, there is a need to develop cost-effective alternative HPV vaccines, for use particularly in developing countries.

In previous studies, plant-made HPV 16 VLP-based vaccine candidates have been shown to induce neutralizing antibodies against type-specific HPVs (Chabeda *et al.*, 2019, Fernández-San Millán *et al.*, 2008, Maclean *et al.*, 2007). Furthermore, it was shown that CRPV challenged rabbits were protected against experimentally induced papillomas, after vaccination with TMV encompassing L2 epitopes (Palmer *et al.*, 2006), and CRPV L1 protein (Kohl *et al.*, 2006). These data suggest that plants have the potential to produce effective papillomavirus vaccines.

Neutralizing antibodies are considered the main immune mechanism of protective responses against HPV infections (Pinto *et al.*, 2018, Kemp *et al.*, 2011). Therefore, HPV vaccine candidates should be able to elicit neutralizing antibodies. The development of PsVs have allowed researchers to measure the neutralization of HPV infectivity both *in vivo* and *in vitro*. PsVs are currently produced by transfecting HEK293TT cells with codon optimized HPV L1 and L2 genes, and a reporter gene (SEAP). These cells are known to over-express simian virus 40 (SV40) large T antigen, which enable reporter plasmid replication, and eventually increased PsVs production (Buck *et al.*, 2005a). Subsequently, PsVs are used in HPV PBNAs (Buck *et al.*, 2005a), which are considered as standard assessment for the immunogenicity of HPV vaccines candidates (Lamprecht *et al.*, 2016).

The usage of PsVs in neutralization assays is based on the ability of PsVs to deliver plasmid DNA into cell lines and tissues as an innate virion would (Rossi *et al.*, 2000). However, producing PsVs in mammalian cells is a highly expensive system which ultimately might be a major drawback in the production of cheaper HPV vaccines for use in developing countries. Our group was the first to make PsVs in plants as a cost-effective alternative. Plant-produced HPV 16 PsVs had comparable results in PBNAs to PsVs produced in mammalian cells, a proof concept that plants are a promising cost-effective system for the production of PsVs (Lamprecht *et al.*, 2016).

Efforts toward the production of affordable HPV vaccines in plants mainly focused on the oncogenic HPV 16, which has been shown to elicit strong immune response an animal model. To date, the antigenicity of plant-produced VLP-based vaccine of other

high-risk HPV types has not been evaluated. The production of cost-effective HPV vaccines for use in resource-poor regions, particularly Africa was of the main interest in this study. This chapter assessed whether plant-produced VLPs of high-risk HPV 35, 52 and 58 induced humoral immune response as effectively as yeast produced VLPs (Gardasil®). Furthermore, the chapter fits in with another greater aim of the project, which is to obtain spleens from hyper-immunized (a prolonged immune-response induction) mice, to produce a mouse anti-HPVs antibody library. However, this study just went as far as hyper-immunizing mice and testing the immune response.

In conclusion, mice were hyper-immunized with Gardasil® and pooled plant-produced VLPs of HPV 35, 52 and 58. The immunogenicity of the VLP-based vaccine candidates was evaluated by detecting type-specific L1s in ELISAs and western blots using sera from immunized mice. Sera from immunized mice were also tested for type-specific anti-L1 neutralizing antibodies using PBNAs. Furthermore, sera from mice hyper-immunized with plant-produced VLPs were tested for the ability to cross-neutralize heterologous PsVs that were available in our lab.

3.2 Materials and methods

3.2.1 Mice studies

This immunogenicity study was approved by the Animal Research Ethics Committee at the Faculty of Health Sciences at the University of Cape Town (UCT, AEC 018-024). The BALB/c mice used in this study were bred at the UCT Animal Research Unit, all handling and procedures done on the animals were done by a registered and experienced animal technologist, Rodney Lucas at the Animal Research Unit. Fifteen 8-week-old female mice were randomly divided into 3 groups of 5 each and housed in the biosafety level II (BSL-2) animal laboratory. Mice were hyper-immunized with Gardasil® (positive control), plant-made VLPs or purified empty vector (prepared in chapter 2) (Table 3.1). The plant-made vaccine candidates and empty vector negative control were emulsified in mineral oil MONTANIDE™ ISA 50 V2 adjuvant at a 60:40 (vaccine: adjuvant) ratio. MONTANIDE ISA 50 V2 is a ready-to-use oily vaccine adjuvant that has previously been used in mice (Cangussu *et al.*, 2018, De Vleeschauwer *et al.*, 2018).

Table 3.1: Vaccine candidates used to immunize mice

Vaccine group	Vaccine name	HPV L1 content	Adjuvant	# of mice	L1 dose µg/100µl	Total L1 dose µg/100µl
Group 1	Gardasil®	6, 11, 16 and 18	Amorphous aluminum hydroxyphosphate sulfate	5	HPV 6 - 4µg HPV 11-8µg HPV 16 -8µg HPV 18 -4µg	24µg (1/5th human dose)
Group 2	Plant-made VLPs	35, 52 and 58	MONTANIDE™ ISA 50 V2	5	HPV 35 -2µg HPV 52 -2µg HPV58 - 2µg	6µg
Group 3	Plant purified empty vector	-	MONTANIDE™ ISA 50 V2	5	-	-

Mice were acclimatised in the BSL-2 laboratory for 7 days prior to any experimental procedures. Three days prior to the first immunization (day 0), pre-bleeds were collected from individual mice. Bleed samples were collected from the tail artery using a 25G needle. Mice were subcutaneously injected with 50µl into both the left and the right flank on day 3, 17, 31 and 45 (every 2 weeks), before obtaining test bleeds on day 56. An additional boost inoculation was administered on day 59, before obtaining final bleeds and spleens on day 73. Here, mice were euthanised by exsanguination under general anaesthesia. A combination of ketamine/xylazine (200µL per 20g mouse) was administered via intraperitoneal injection using a 25G needle. After the depth of anaesthesia was confirmed by pedal reflex, mice were placed in dorsal recumbency, and a 25G needle was inserted adjacent the xiphoid cartilage and into the heart. A maximum of 1.8mL of blood was then collected and stored at 4°C. Mice were hyper immunized in order to obtain high affinity binders to HPVs, which was one of the goals of this project. Blood was collected in capillary blood collection tubes (Impromini®) and serum isolated by centrifugation at 10 000 x *g* for 10 minutes and stored at -20°C. Additionally, spleens from hyper-immunized mice were preserved in RNAlater (Thermo Fisher Scientific) at 4°C for overnight to stabilize and protect RNA, before stored at -80°C.

3.2.2 Detection of anti-L1 antibodies in mice sera

3.2.2.1 Pre-absorption of mice sera

Purified plant-produced HPV L1 proteins (made in chapter 2) were used as antigens in both western blots and ELISAs. It has previously been reported that host cell proteins (in this case co-purified plant proteins) will also result in the production of antibodies against these proteins that are present in the plant-made vaccine candidates (Chabeda *et al.*, 2019, Pineo *et al.*, 2013). This was confirmed by initial ELISAs carried out on test and final bleeds which showed high background detection of plant proteins in the empty vector serum. Furthermore, the detection of plant-made L1 using final bleeds of plant-produced VLPs and empty vector resulted in the detection of non-specific bands with high band intensities on western blots.

To reduce the cross-reactivity of the antibodies present in the sera with host cell proteins the final bleeds from mice immunized with plant-made L1 and plant-purified empty vector were pre-absorbed using The “Lunchbox” Immunoabsorbent Technique

(Rybicki 1990, 2000: http://www.mcb.uct.ac.za/mcb/resources/molbio_techniques/lunchbox) (Rybicki *et al.*, 1990), to potentially remove/reduce antibodies against native plant proteins present in the mice sera. Briefly, 6g of un-infiltrated plant leaves were homogenised in 2x HSPBS (pH 7.4) using mortar and pestle. The crude extract was filtered through 4 layers of 22-24µm pore Miracloth™ (MilliporeSigma) to remove plant debris. Nitrocellulose membrane was incubated with the crude plant extract at 37°C for 1 hour with agitation. The membrane was washed 4x with blocking buffer (5% long life fat-free milk, 1x Tris-Cl [pH7.5]) before incubation in 1:200 diluted mice serum overnight at 4°C. The following day, the pre-absorbed sera were removed and used in all ELISAs and western blots analysis in this study.

3.2.2.2 ELISAs and western blots analysis

For ELISAs, 96-well plates (Thermo Fisher Scientific) were coated with antigens diluted to 80ng/100µl in coating buffer (10Mm Tris pH 8.5) before incubation overnight at 4°C with agitation. Plates were blocked with 300µl blocking buffer (5% long life fat-free milk, 1x Tris-Cl pH7.5) for 1 hour at 37°C, before they were washed 4x with 1x TST (0.05% tween 20, 1xTris-Cl pH7.5). Mice sera or rabbit-raised anti-Gardasil® antibody at the desired dilution were added to the wells and the plates incubated for 1 hour at 37°C. The plates were washed as described above and 100µl of 1:5000 alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich®) added to each well and the plate incubated for 1 hour at 37°C. Plates were washed 4x with (1x Tris-Cl pH 9.0) after which 200µl SIGMAFAST™ *p*-nitrophenyl phosphate (Sigma-Aldrich®) substrate was added to each well. The plates were incubated in the dark for 30 minutes before reading the absorbance using Bio-Tek Powerwave XS spectrophotometer at 405nm.

Controls included: wells coated with coating buffer (to obtain background readings), pre-bleeds (negative controls) and rabbit-raised anti-Gardasil® antibody (positive control). Plant-produced empty vector was used as negative control for plant-made vaccine candidates only.

ELISA data were all normalised by subtracting the background readings and titres are stated as the reciprocal of the maximum dilution with higher absorbance readings than the equivalent pre-bleed serum. Moreover, for plant serum, titres with absorbance

readings that were higher than the empty vector at the lowest dilution (1:200) were considered anti-L1 positive. ELISA experiments were repeated 3 times, and a representative of the 3 experiments for each HPV type is reported here.

Western blots were performed as described in Chapter 2, Section 2.2.9. Pooled mice sera were used as primary antibody at a dilution of 1:1000, alkaline phosphatase-conjugated anti-mouse IgG antibody (Sigma-Aldrich®) diluted at 1:5000 was used as a secondary antibody. Pre-bleeds were used as negative control serum, with an additional empty vector control for plant-made vaccine candidates. Rabbit-raised Gardasil® antiserum (1:2000) was used as positive antibody control and was detected with secondary alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich®) (1:5000).

3.2.2.3 Statistical analysis

Quantitative ELISA data were assessed using a two-tailed t-test for experimental and control groups. Furthermore, One-way analysis of variance (ANOVA) test was performed to assess the difference among HPV types. Significance threshold (*p* value) of 5% ($p = 0.05$) was used (Charan and Kantharia, 2013).

3.2.3 Detection of anti-L1 neutralizing antibodies (NAb) in mice sera

3.2.3.1 HPV PsVs production in HEK293TT cells

HPV 6, 31, 45, 52 and 58 PsVs were kindly donated by our lab (made by Megan Hendrikse and Cathy Pineo), while HPV 16, 18 and 35 PsVs were made in this study. Recombinant *E. coli* harbouring the HPV plasmids (p16 shell, p18 shell, and pshell 35) containing both L1 and L2, and pYSEAP (reporter gene plasmid) were obtained from Dr John Schiller (National Cancer Institute). HPV and pYSEAP clones were selected on media containing ampicillin (100µg/ml) and blasticidin (75µg/ml). Plasmid DNA was extracted using endotoxin free PureYield™ plasmid Maxiprep kit (Promega) and stored at -20 °C until transfection of HEK cells.

3.2.3.2 HEK293TT cells culturing and transfection

PsVs were made as described in the pseudovirus production protocol of the Laboratory of Cellular Oncology https://home.ccr.cancer.gov/lco/pseudovirusproduction.htm (Buck *et al.*, 2005b). Cells

were cultured in 1x complete Dulbecco's Modified Eagle Medium (cDMEM) and 1x GlutaMAX^{TM}-I (Gibco). The medium was supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 250µg/ml Hygromycin B (Roche). Cells were cultured in pyrogen-free Corning cell culture flasks and unless otherwise stated, incubated in a 37°C, 5% CO_2 incubator.

For transfection, cells that reached 60-70% confluency, were transfected by mixing 37.5µg of HPV plasmid DNA and 37.5µg of pYSEAP DNA into 3ml of serum-free DMEM medium. A volume of 100µl X-tremeGENE HP DNA transfection reagent (Roche) was added and the medium incubated at room temperature for 15 minutes. The transfection mixture was then added onto cells and incubated for 40-48 hours.

3.2.3.3 Collection, maturation and purification of PsVs

Approximately 40-48 hours post-transfection, cells were harvested by centrifugation for 2 minutes at 832 x *g* using 221.08 V01 rotor (Beckman). Briefly, culture medium was removed and added into a sterile conical tube and centrifuged as described above. After centrifugation the supernatant was discarded. Cells attached to the flasks were removed by adding 0,005% trypsin-EDTA (Gibco) and incubated for approximately 5 minutes at 37°C. The enzyme reaction was deactivated by adding cDMEM medium, and the resuspension added to the pellet obtained after centrifugation of the media. Cells were collected by centrifugation as above. The supernatant was aspirated and discarded, and the pellet washed with 0.5mL Dulbecco's phosphate buffered saline (DPBS) (Invitrogen), centrifuged and the supernatant discarded.

For maturation of PsVs, the pellet was partially resuspended in DPBS and transferred into a 2ml siliconized-microcentrifuge tube. DPBS was removed by centrifugation, the pellet volume was estimated, and the pellet was resuspended in 1.5 volumes of DPBS-9.5mM $MgCl_2$; 1/20th volume of 10% triton X-100; 1/40th of 1M ammonium sulphate (pH 9.0) and 1/1000th of RNase (Ambion). The resuspension was gently mixed by tapping the microcentrifuge tube and incubated at 37°C overnight.

The following day, the matured PsVs were purified. Briefly, the microcentrifuge tube with cell resuspension containing matured PsVs was incubated on ice for 5 minutes, before centrifugation using a Benchtop centrifuge (Thermo Fisher Scientific) at 5000 x *g* for 5 minutes. The supernatant was collected in a fresh siliconized tube and pellet

washed in 2x volume DPBS and centrifuged again. The supernatant was added to the first supernatant collected and the washing step was repeated twice more, using one-pellet volume for both the first and second wash, with the second wash step supplemented with 0.8M NaCl. Supernatants collected from all the washing steps were combined and centrifuged again before purification by ultracentrifugation. For purification, the supernatant was loaded into Optiprep™ discontinuous gradients (1ml 27%, 1ml 33% and 1ml 39%) and centrifuged for 4.5 hours at 234 843 x *g*, 15°C using a SW 55 Ti rotor (Beckman). After centrifugation 250µl factions were collected from the bottom of the tubes by puncturing the lowermost part of the tube.

3.2.3.4 Analysis of HPV PsVs

Fraction 4 -12, where PsVs were expected to sediment were analysed on dot blots using conformational type-specific monoclonal antibodies (MAbs) from Dr Neil Christensen (Penn State Cancer Institute) at a dilution of 1:1000 (Table 3.2) and anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich®) at 1:5000. Dot blots were performed as described in Chapter 2, Section 2.2.11.1.

Table 3.2: Type-specific monoclonal antibodies

HPV type	MAbs
HPV 16	H16V5
HPV 18	H18.J4
HPV 35	H35Q8

Successful encapsidation of the secreted alkaline phosphatase reporter plasmid by PsVs was assessed by incubating PsVs (1:100) with HEK293TT cells for ~72 hours and SEAP signal determined. Briefly, cells that reached 60-70% confluency were collected by adding 0,005% trypsin for 5 minutes, before neutralizing with complete medium. Cells were collected by centrifugation for 2 minutes at 832 x *g* using 221.08 V01 rotor (Beckman) and resuspended in fresh medium, after which they were diluted to 3 x 10^5 cells/ml. Cells were then seeded (100µl/well) in pyrogen-free 96-wells tissue culture plates (Sigma-Aldrich®) and left to attach by incubating the plates for at least 4 hours. The outer wells were unused to avoid evaporation and were filled with medium.

A volume of 100µl/well of PsVs were added onto cells in duplicate (due to the high cost of the SEAP kit).

Controls included: cells only (to obtain background readings) and cells transfected with pYSEAP plasmid (SEAP positive control). Plates were incubated for 72 hours, after which SEAP were signals detected.

SEAP signals were determined using a Great Escape kit 2.0 (Clontech Laboratories, Inc) according to the manufacturer's instructs, with a few adjustments. The volume used in the assays in this study were 0.6 volumes that of the recommended volumes in the neutralization assay manual of the Laboratory of Cellular Oncology. Briefly, 15µl of infected cells were transferred into corresponding wells of a white 96-well plate (Thermo Fisher Scientific) and 45µl 1x dilution buffer was added. The plate was covered and incubated at 65°C for 30 minutes, after which the plate was chilled on ice for 5 minutes before adding 60µl SEAP substrate. The plate was incubated at room temperature for 45 minutes before SEAP signal determined using GloMax®-Multi Detection System (Promega).

PsVs fractions with the highest SEAP signals and amount of L1 (based on the dot blots) were pooled and analysed under TEM (as described in Chapter 2) for the presence of PsVs. PsVs were stored at -80°C until further analysis.

3.2.3.5 Pseudovirion-based neutralization assays

3.2.3.5.1 Optimization of PsVs and Neutralization antibodies dilutions

PsVs were initially tested at different dilutions to determine the dilution at which to use them in neutralization assays. Briefly, cells were grown until they were 60-70% confluent and diluted to 3 x 105 cells/ml. Cells were then seeded (100µl/well) in pyrogen-free 96-wells tissue culture plates (Sigma-Aldrich®) and left to attach by incubating the plates for about 4 hours. A dilution range of 1:250-1:1000 of PsVs were prepared in sterile conical tubes using complete DMEM medium. PsVs dilutions were added onto cells (100µl/well) in duplicate. Cells only were used to get background readings and 100µl medium was added to these wells. Plates were incubated for 72 hours in the incubator before analysing SEAP signals as described in Section 3.2.3.4. Data were normalised by subtracting the cells only data, and the minimum dilution that

gave a robust SEAP signal (at least a reading of 2 million relative light unit (RLU) was selected to be used in the assays.

Furthermore, known type-specific NAbs were tested to determine the dilution that completely neutralized PsVs. Here, the dilution of PsVs that was chosen to be used in PBNAs was prepared for each HPV type. NAbs working stocks were then prepared in fresh DMEM medium before they were added directly to the PsVs to dilute to the desired dilutions (Table 3.3). The dilution ranges were selected based on previous titrations done by our group. Antibodies were left to attach to PsVs for at least 1 hour at 4°C before adding onto cells. Controls included: cells only (background readings) and PsVs only (no neutralization).

Plates were incubated for 72 hours before analysing SEAP signals as described in Section 3.2.3.4. Data were normalised by subtracting cells only readings. PsVs only data were then set as maximum (100%) SEAP signal and the dilution that reduced SEAP signal by ~100% when compared to PsVs only control was selected to be used in PBNAs.

Table 3.3: Type-specific neutralizing antibodies used in PBNAs

NAb Name	HPV type	Dilution range	Fold increase
H16V5	HPV 16	$5x10^3$-$2x10^4$	2
H18.J4	HPV 18	$1x10^4$ and $2x10^4$- $2x10^6$	2 and 10
H35Q8	HPV 35	$5x10^2$- $5x10^5$	10

3.2.3.5.2 Neutralization assays

L1-neutralization assays were done as described in the Laboratory of Cellular Oncology files https://ccrod.cancer.gov/confluence/display/LCOTF/NeutralizationAssay (Buck *et al.*, 2005b). Briefly, cells were grown as above until they reached 60-70% confluency and diluted to 3 x 10^5 cells/ml. Tissue culture plates were seeded with 100µl/wells and incubated for 4 hours. PsVs, type-specific NAbs and mice sera working stocks were each diluted in fresh medium. Pooled mice sera and NAbs were added directly onto PsVs to reach desired dilutions. PsVs with serum or NAbs were incubated for 1 hour

before being added onto cells and incubated for 72 hours. SEAP signals were detected as described in Section 3.2.3.4.

Controls included were: Cells only (background readings); PsVs only (no neutralization); PsVs pre-incubated with known NAbs (positive neutralizing antibody control); PsVs pre-incubated with empty vector serum (negative neutralizing antibody control for plant serum).

Empty vector serum was used at a one-point dilution (lowest dilution). All raw data were normalised for background by subtracting the cells only data, thereafter PsVs only data were set as the maximum (100%) SEAP signal. For each HPV type, neutralization is expressed towards its own PsVs only control, and data plotted as percentage relative light unit (% RLU). The assays were repeated 3 times, and a representative assay for all the 3 experiments of each type is reported here.

3.2.3.6 Statistical analysis

One-way ANOVA test was performed to assess the difference in neutralization ability among HPV types, with a significance threshold (p value) of 5% ($p = 0.05$) (Charan and Kantharia, 2013).

3.3 Results

3.3.1 Detection of anti-L1 antibodies in mice sera

3.3.1.1 ELISA analysis

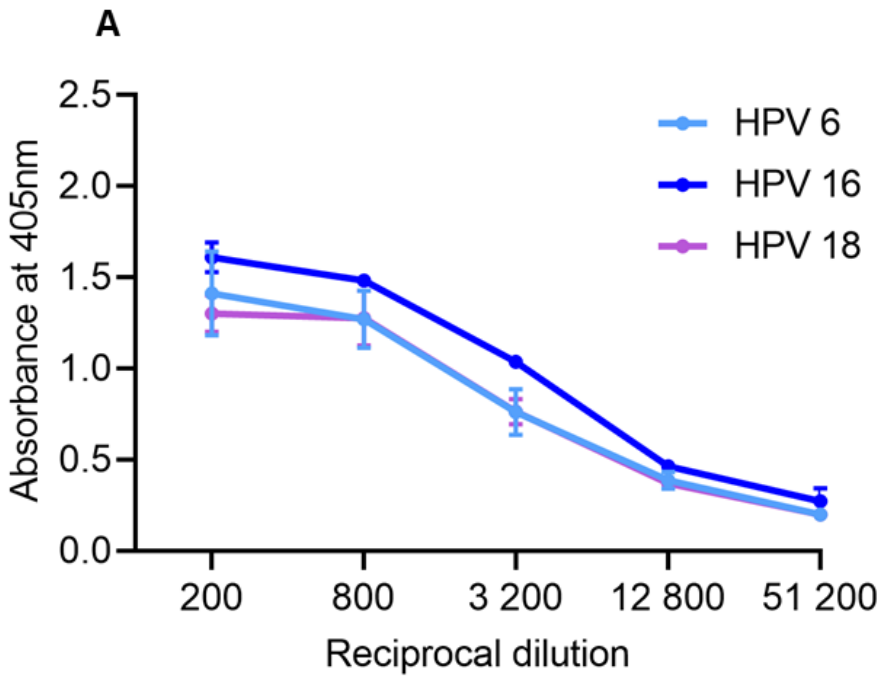
To determine whether plant-produced HPV VLPs can elicit a humoral immune response in mice as efficiently as the commercially available Gardasil®, mice were hyper-immunized with a total of 6µg of plant-produced VLPs or 1/5th of human dose of Gardasil® (total amount of 24µg). The humoral immune response was analysed by testing type specific anti-L1 antibodies in ELISA using plant-produced VLPs as antigen.

The first step was to determine if there were any non-responders to the vaccine candidates. Sera from individual mice inoculated with plant-made VLPs and Gardasil® were tested against HPV 58 and HPV 16 antigens, respectively. These HPV types were chosen from each group to avoid wasting sera by testing individual mice on each antigen. Serum from mice inoculated with empty vector, served as a negative control to which serum against plant-produced VLPs could be compared, these sera were tested against HPV 58 only. All sera were tested at 1:200 dilution and successfully detected corresponding L1 proteins (results not shown). Sera from individual mouse in each group were then pooled for further analysis.

Pooled sera were titrated using each of the HPV types included in the vaccine candidates to determine the anti-L1 endpoint titres. Sera were titrated using a 4-fold serial dilution ranging from 1:200-1:51200 dilution. Serum collected from mice vaccinated with Gardasil® was only tested on HPV 6, 16 and 18 VLPs as we do not have the HPV 11 L1 gene with which to produce HPV 11 VLPs, therefore it was excluded in this study. The empty vector serum and serum obtained from plant-made VLP-vaccinated mice were tested against all 3 HPV types (HPV 35, 52 and 58) included in the plant-made vaccine candidate. Pre-bleed sera served as negative control against the corresponding HPV types and were tested at the lowest dilution (1:200). Rabbit-raised anti-Gardasil® antibody (1:5000) served as a positive control that confirmed the presence of antigens in the coated samples and validated the experiments. Anti-L1 titres are stated as the reciprocal of the maximum dilution with higher absorbance readings than the equivalent pre-bleed serum.

Gardasil® serum

The commercial Gardasil® vaccine elicited a significant immune response, with the highest anti-L1 titre of 51200 observed for all three antigens tested (Figure 3.1A). It is also important to note that, Gardasil® serum detected HPV 16 L1 better when compared to HPV 6 and 18. This was expected, as Gardasil® contained more HPV 16 L1 (8µg) compared to HPV 6 and 18 (4µg) per dose (1/5th human dose was used). However, regardless of the different amounts of L1 VLPs in the vaccine dose, no statistically significant difference was observed in the immune response elicited against these HPV types (p=0.8351). As expected, no anti-L1 response observed in the pre-bleed serum from mice inoculated with Gardasil® which served as a negative control (Figure 3.1B). Rabbit-raised anti-Gardasil® successfully detected all HPV VLPs which validated the experiments (Figure 3.1C).



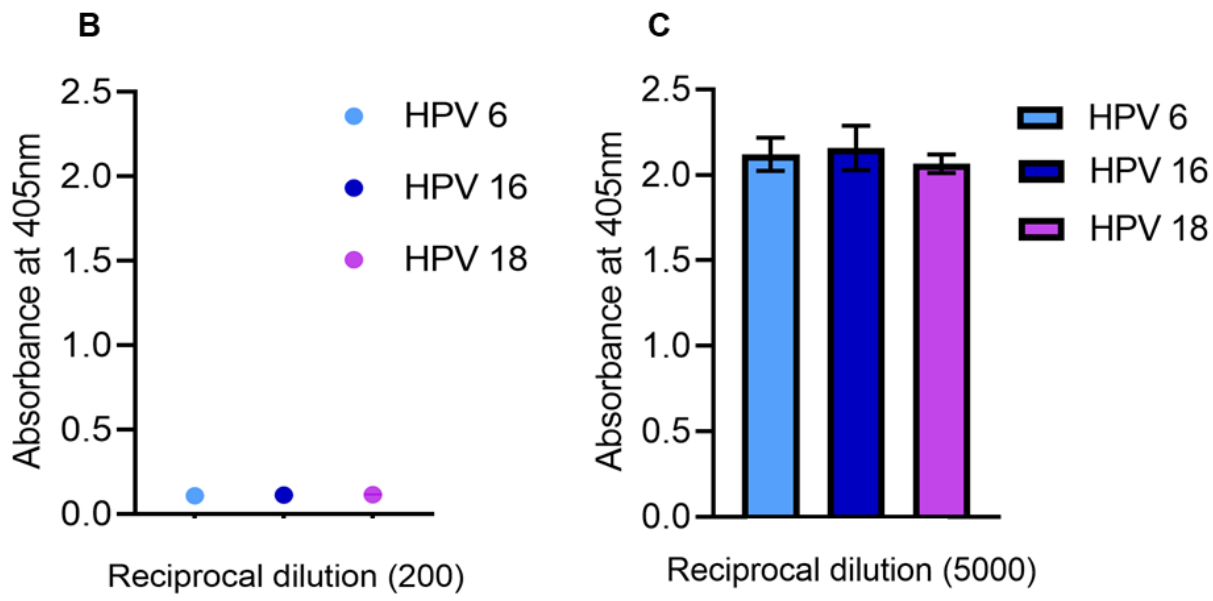
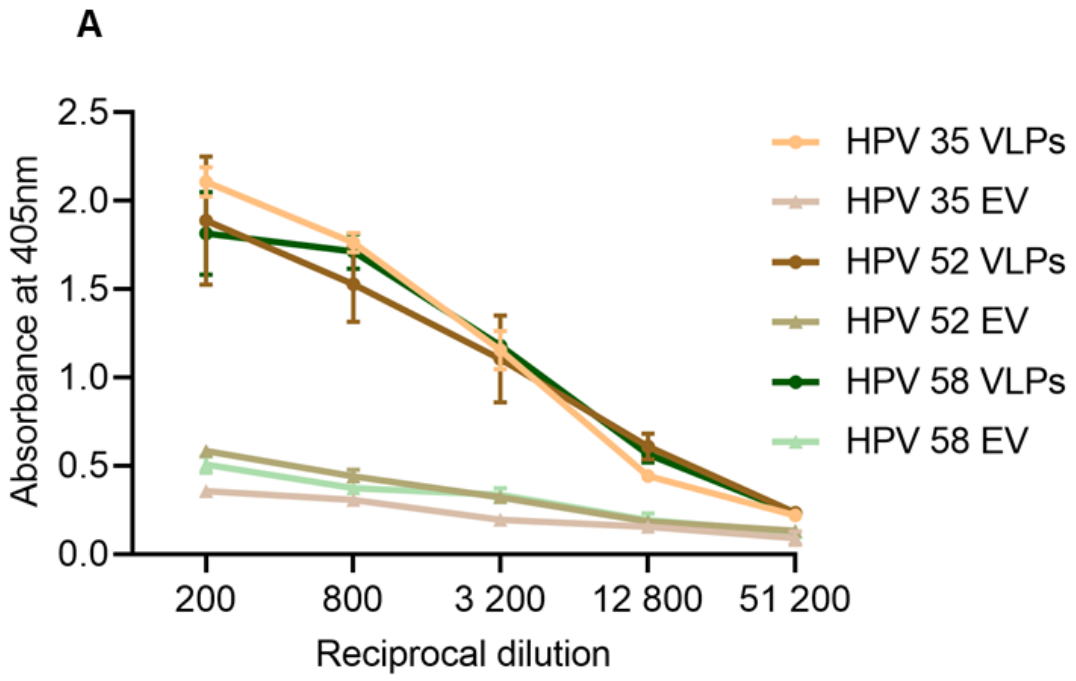


Figure 3.1: Indirect ELISA detection of anti-L1 in Gardasil® serum using plant-made L1 coating antigens: A) Four-fold serial dilution of pooled Gardasil® serum B) Absorbance values obtained for pre-bleeds at 1:200 dilution C) Rabbit-raised anti-Gardasil® positive control at 1:5000. Markers and error bars indicate mean values and standard deviation from triplicate readings.

Serum from mice immunized with plant-produced VLPs

High background signals were obtained for the negative control empty vector serum and serum from mice immunized with plant-produced VLPs, these results were consistent with the ELISA results from the test bleeds at day 56 of vaccination (results not shown). Higher absorbance readings observed possibly resulted from antibodies raised against co-purified host cell proteins, which have been reported before (Chabeda *et al.*, 2019, Pineo *et al.*, 2013). Pooled sera were pre-absorbed against plant proteins at a dilution of 1:200, to potentially remove antibodies against host cell proteins. Pre-absorption of sera successfully removed most antibodies against plant contaminants. This was evident by the decrease in absorbance values obtained for ELISAs, especially for the negative control group after pre-absorption. The highest absorbance values before pre-absorption were ~1 and 3 for the empty vector and plant-produced VLPs serum, respectively. After pre-absorption, these values dropped to ~0,5 and 2, indicating successful reduction of antibodies against native plant proteins (Figure 3.2A).

Plant-produced L1 VLPs elicited a significant humoral immune response against type-specific HPVs (Figure 3.2A). The highest anti-L1 titres of 12800 was observed against all the 3 HPVs (Figure 3.2A). The immune response elicited against HPV 35, 52 and 58 were comparable to each other and the difference in immune response observed between these HPV types was not statistically significant (p= 0.9900). Titres of 200-3200 were observed for the empty vector, which may have been due to antibodies against plant contaminants (Figure 3.2A). This suggested that pre-absorption did not remove all the antibodies against host cell proteins. The immune response against HPV 35, 52 and 58 were statistically significant when compared to the empty vector control (p=0,0372; p=0,0443 p=0.0371, respectively). As expected, pre-bleeds negative controls from mice inoculated with VLPs and empty vector showed no anti-L1 titres for all three HPV types (Figure 3.2B). The positive control rabbit-raised anti-Gardasil® antibody successfully detected all HPV types, which validated these experiments (Figure 3.2C).



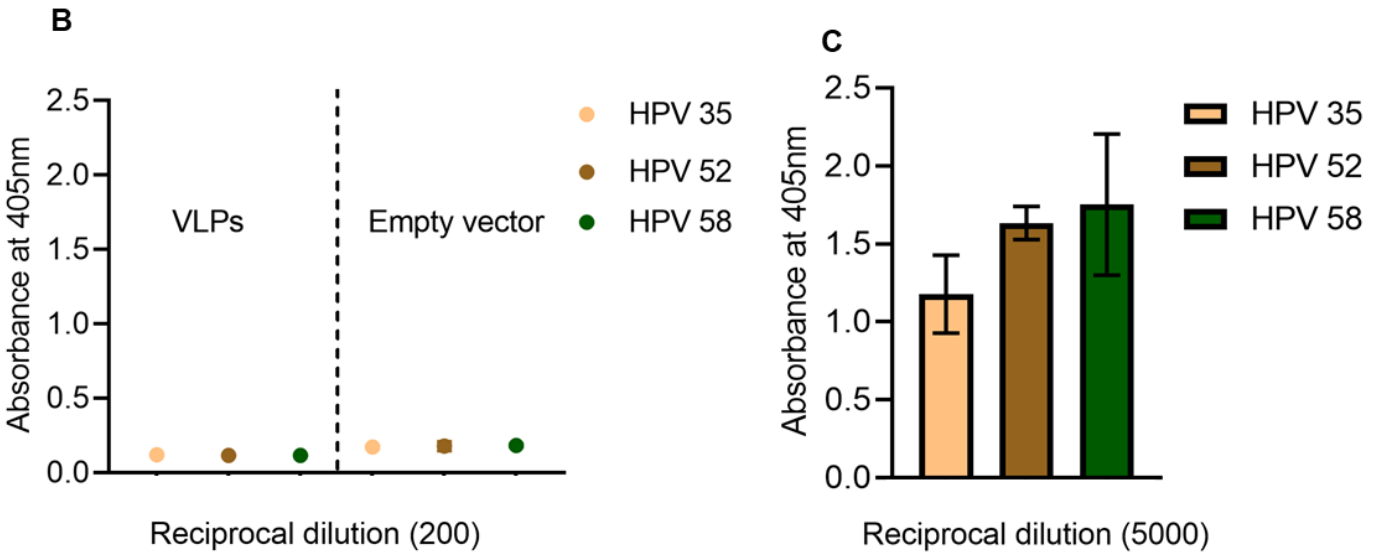


Figure 3.2: Indirect ELISA detection of anti-L1 in plant serum using plant-made L1 coating antigens: A) Four-fold serial dilution of pooled plant serum B) Absorbance values obtained for pre-bleeds at 1:200 dilution C) Rabbit-raised anti-Gardasil® positive control at 1:5000 dilution. Labels: EV= empty vector. Markers and error bars indicate mean values and standard deviation from triplicate readings.

3.3.1.2 Western blot analysis

After successfully detected anti-L1 antibodies in ELISAs, the ability of sera from immunized mice to detect denatured L1 proteins was assessed by western blot analysis using plant-made antigens and mammalian-made antigens (in the case of sera from mice immunized with plant-made VLPs). Pooled final bleeds and pre-bleed sera of all vaccine groups were analysed for corresponding type-specific anti-L1 immune responses. Rabbit-raised anti-Gardasil® antibody obtained from our lab was used a positive antibody control.

Gardasil® serum

Gardasil® serum successfully detected type-specific HPV L1 proteins at ~56 kDa and is validated by the positive anti-Gardasil® antibody control which detected the same bands (yellow arrows, Figure 3.3A and B). As expected, no L1 proteins were detected by the pre-bleed negative control (Figure 3.3C).

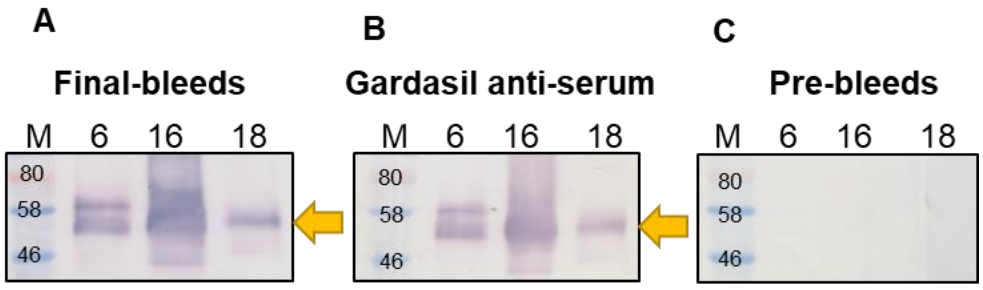


Figure 3.3: Anti-L1 western blot analysis with Gardasil® serum: A) Final bleeds B) Rabbit-raised anti-Gardasil® positive control C) Pre-bleeds. Labels: M= pre-stained protein standard (kDa). L1 proteins (~56 kDa, yellow arrow) were detected with either final bleeds, pre-bleeds (1:1000) or anti-Gardasil® (1:2000) and anti-mouse or anti-rabbit IgG alkaline phosphatase-conjugated secondary antibodies (1:5000).

Serum from mice immunized with plant-produced VLPs

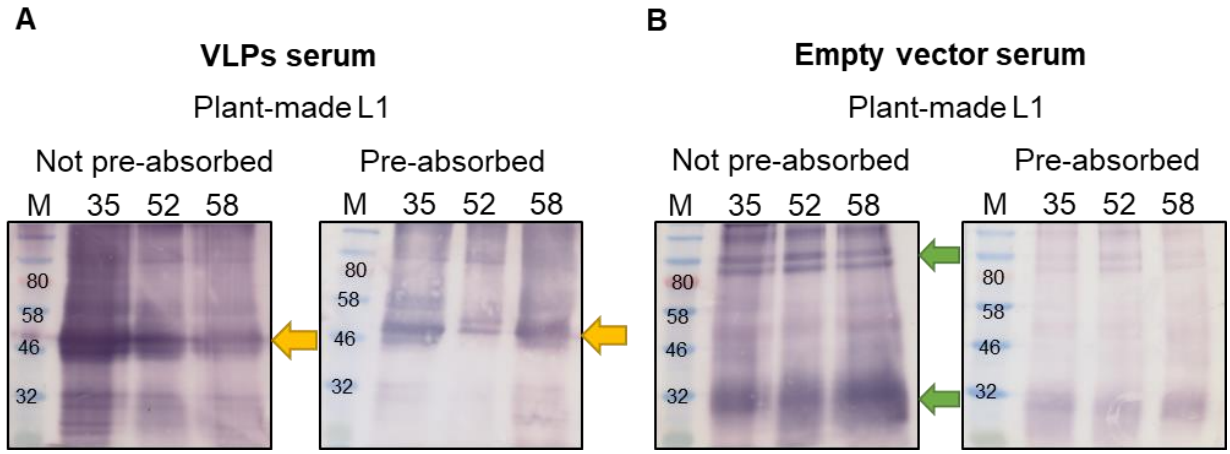
Initially western blots were carried out using sera that was not pre-absorbed with host cell proteins. Plant-produced VLPs serum successfully detected type-specific HPV L1 at ~56 kDa (yellow arrow), with no L1 band observed in the empty vector serum (Figure 3.4A and B, respectively). However, high background was obtained when probing plant-produced VLPs with non-pre-absorbed serum from this study, as evidenced by the detection of smears and non-specific bands (~82 and 32 kDa) with high intensity on western blots, especially in the empty vector control sample (green arrows, Figure 3.4B). This possibly resulted from antibodies raised against co-purified host cell proteins. Therefore, western blots were further performed using pre-absorbed sera. Pre-absorbed plant serum successfully detected type-specific HPV L1 proteins at ~56 kDa (yellow arrow, Figure 3.4A). As expected, no L1 proteins were detected by pre-absorbed empty vector serum (Figure 3.4B).

By comparing the blots detected using pre-absorbed serum with those detected with serum that was not pre-absorbed, it was evident that pre-absorption of sera sucessfully removed most of the antibodies against host proteins. Non-specific binding was still obtained with the pre-absorbed sera, however the intensity of background signal and bands detected was much lower compared to non-pre-absorbed sera (Figure 3.4A and B), indicating the successful removal of most of the host-cell-specific antibodies. These results are consistent with those observed in ELISA experiments, where a decrease in absorbance values was observed after using pre-absorbed

serum. The low background observed when using pre-absorbed plant-produced VLPs and empty vector sera further suggested that pre-absorption did not remove all the antibodies against host cell proteins.

Since pre-absorption of sera did not remove all the antibodies against host cell proteins, serum from mice inoculated with plant-made VLPs was futher tested to see whether they will detect mammalian-made L1 in type-specific PsVs used in this study. Mammalian-made L1s were detected using non-pre-absorbed serum. As expected the serum successfully detected HPV L1 proteins at ~56 kDa (yellow arrows) with no no-specific bands and/or background signal observed (Figure 3.4C). Furthermore, a band at ~46 kDa (black arrow) was observed for HPV 52 L1, this might have been a product of protein cleavage, which possibly happened during storage (Figure 3.4C). The ability of plant serum to successfully detect mammalian-made L1s with non-specific binding demostrated the novelty of anti-serum obtained in this study. This confirmed that the background signals detected when using plant-made antigens are against plant contaminants that were present in vaccine samples.

The positive control, rabbit-raised anti-Gardasil® antibody, successfully detected L1 proteins at ~56 kDa (yellow arrow, Figure 3.4D). This validated the western blot experiments. As expected, no L1 proteins or background were observed in the pre-bleed negative controls of both VLPs and empty vector vaccinated groups (Figure 3.4E and F). This further confirmed that the background signals detected in western blots are against plant contaminants that were present in vaccine samples.



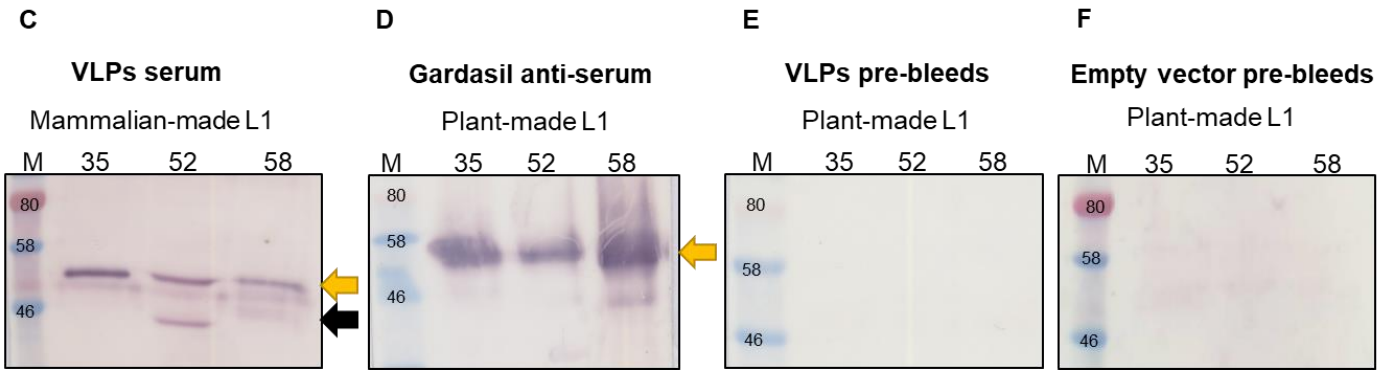


Figure 3.4: Anti-L1 western blot analysis with serum from plant-produced VLPs: A) Plant-made antigens with non-pre-absorbed and pre-absorbed VLPs sera and empty vector sera (B); C) Mammalian-made antigens with non-pre-absorbed VLPs serum; D) Rabbit-raised Gardasil® anti-serum; E and F) VLPs and empty vector pre-bleeds, respectively. Labels: M= pre-stained protein standard (kDa). L1 proteins (~56 kDa, yellow arrows) were detected with either VLPs final bleeds, empty vector final bleeds and pre-bleeds serum (1:1000) or anti-Gardasil® (1:2000) and anti-mouse or anti-rabbit IgG alkaline phosphatase-conjugated secondary antibodies at 1:5000.

3.3.1.3 Overview of type-specific anti-L1 titres of mice sera

Mice were hyper-immunized with Gardasil® and plant-produced fully assembled VLPs with aggregates and capsomeres (Chapter 2, Figure 2.6, Section 2.3.4). Purified empty vector was used as a negative control for plant-produced vaccine candidate. Hyper-immunization of mice resulted in a strong humoral immune response, suggesting the presence of L1 epitopes on the surface of L1 particles. Table 3.4 summarizes the anti-L1 immune responses obtained in this study.

Table 3.4: Anti-L1 immune response in mice sera

HPV types	Vaccine group	Anti-L1 titre	Detection of denatured L1
6	Gardasil®	51200	Y
16	Gardasil®	51200	Y
18	Gardasil®	51200	Y
35	Plant-produced VLPs	12800	Y
52	Plant-produced VLPs	12800	Y
58	Plant-produced VLPs	12800	Y
35	Empty vector	None	N
52	Empty vector	None	N
58	Empty vector	None	N

Y= Yes; N=No

3.3.2 Detection of anti-L1 neutralizing antibodies in mice sera

After successfully confirming that plant-produced VLPs and Gardasil® induced type-specific humoral immune responses in mice. The next step was to analyse whether the elicited antibodies can neutralize HPV PsVs in PBNAs and ultimately potentially prevent HPV infections. This was achieved by detecting anti-L1 neutralizing antibodies using PBNAs. Even though the study focused on cost-effective alternatives for HPV vaccine production, mammalian-made PsVs were used in PBNAs instead of the alternative cost-effective plant-produced PsVs. This is because of the non-specific binding to antibodies against plant contaminants observed in ELISAs and western blots experiments (Section 3.3.1.1 and 3.3.1.2, respectively), which I thought might negatively influence PBNAs results for plant-produced VLPs serum.

3.3.2.1 Dot blots analysis of purified PsVs

HPV PsVs made in this study were expressed in HEK293TT cells and purified by Optiprep™ discontinuous density gradient ultracentrifugation. Fractions 4-12 were analysed by detecting L1 proteins with type-specific MAbs on dot blots (Table 3.2,

Section 3.2.3.4). L1 was detected mostly across all the fractions, with strong detection in less dense fractions (F9-12), corresponding to 27% Optiprep™, for HPV 16 (Figure 3.5A). Whereas HPV 18 and 35 L1s were strongly detected in more dense Optiprep™ fractions (F4-8), which corresponds to 33-39% Optiprep™ (Figure 3.5B-C).

It is important to note that, some particles may not contain a reporter plasmid therefore VLPs may be formed instead of PsVs. Fully assembled PsVs encapsidating the reporter plasmid usually sediment in a less dense Optiprep™ fraction as compared to empty particles (Buck *et al.*, 2005a). Therefore, data observed on the dot blots might suggest that HPV 16 made fully encapsidated PsVs as compared to HPV 18 and 35 where L1s were detected in more dense Optiprep™ fractions.

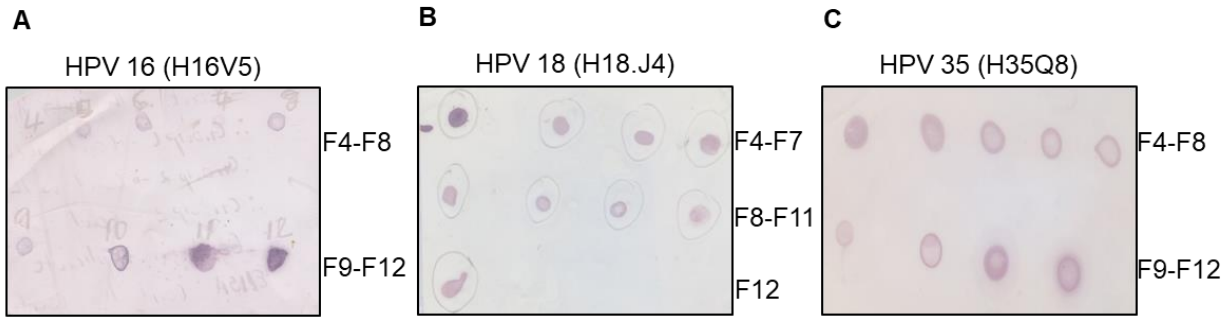


Figure 3.5: Dot blot analysis of purified PsVs: A) HPV 16 PsVs B) HPV 18 PsVs C) HPV 35 PsVs. L1 proteins were detected with type-specific monoclonal antibodies (1:1000) and anti-mouse IgG alkaline phosphatase-conjugated secondary antibodies (1:5000).

To confirm if L1 and L2 assembled into particles with a reporter plasmid. L1-positive fractions were further analysed for SEAP signal at a dilution of 1:100. Fractions showed diverse SEAP signals with better SEAP signals observed for HPV 16 PsVs (results not shown). This further suggested that HPV 16 PsVs encapsidated the reporter plasmid (SEAP) better than those of HPV 18 and 35, which probably had a mix population of VLPs and PsVs. Fractions with the highest SEAP signals and most intense L1 spots observed on dot blots were pooled for TEM and PBNAs analysis.

3.3.2.2 Transmission electron microscopy analysis of purified PsVs

The morphology of purified PsVs was analysed using TEM (Figure 3.6). Fully assembly PsVs (40-60nm) were observed for all HPV types (white arrows, Figure 3.6). HPV 16 showed low yield of fully assembled particles, with small PsVs (35-39nm)

(blue arrow, Figure 3.6A) also observed. Whereas HPV 18 and HPV 45 were entirely populated by fully assembled particles (white arrows, Figure 3.6B and C). It not surprising that small PsVs were observed for HPV 16, as this might have partially contributed to strong detection of L1 in less dense Optiprep™ fractions (Figure 3.5).

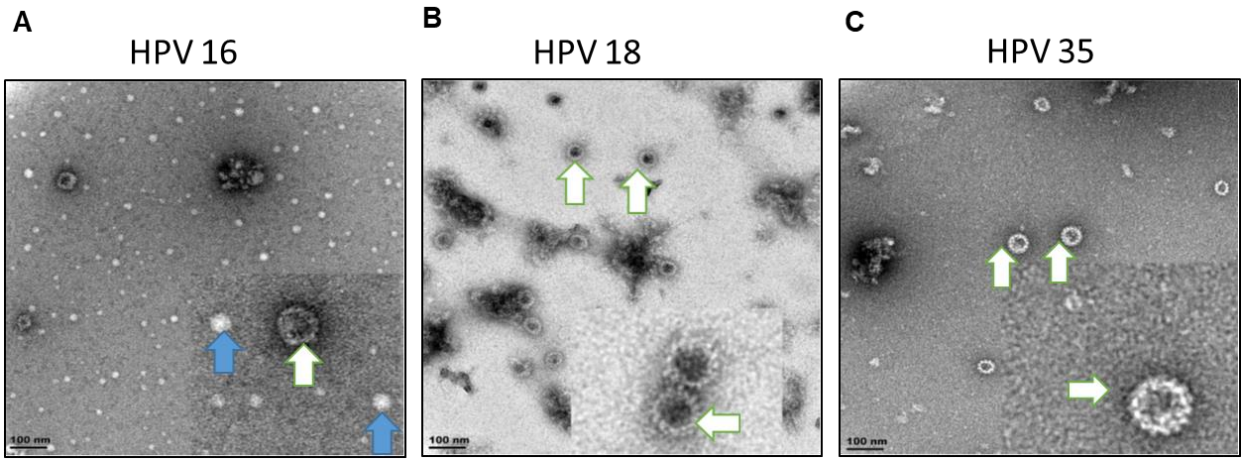


Figure 3.6: TEM micrographs of purified mammalian-produced PsVs: Samples were extracted from HEK293TT cells and purified using discontinuous Optiprep™ gradients and viewed under TEM at the magnification of 40 000 X. Bars are 100nm in size. Labels: white arrows- full assembled PsVs; blue arrows- small PsVs and/or capsomeres.

3.3.2.3 Pseudovirions and neutralization antibodies dilutions selected for PBNAs

Pooled PsVs were initially tested to determine the minimum dilution that gave robust SEAP signals (at least $2x10^6$ RLU readings), which is the dilution used in PBNAs. PsVs were tested using 2-fold dilution ranging from 1:250-1:1000 and based on the SEAP signal, a dilution was selected for each type (red dots, Figure 3.7). For HPV 16 strong SEAP signals were observed at 1:250-1:1000, therefore PsVs were used at 1:1000. On the other hand, HPV 18 and 35 gave robust signal at 1:250-1:500 and PsVs were used at 1:500.

HPV 16 gave better SEAP signal than HPV 18 and 35 (Figure 3.7). This is in consistent with the results obtained from analysing individual PsVs fractions at a dilution of 1:100 (results not shown). This further suggested that HPV 16 PsVs encapsidated the reporter plasmid (SEAP) better than those of HPV 18 and 35.

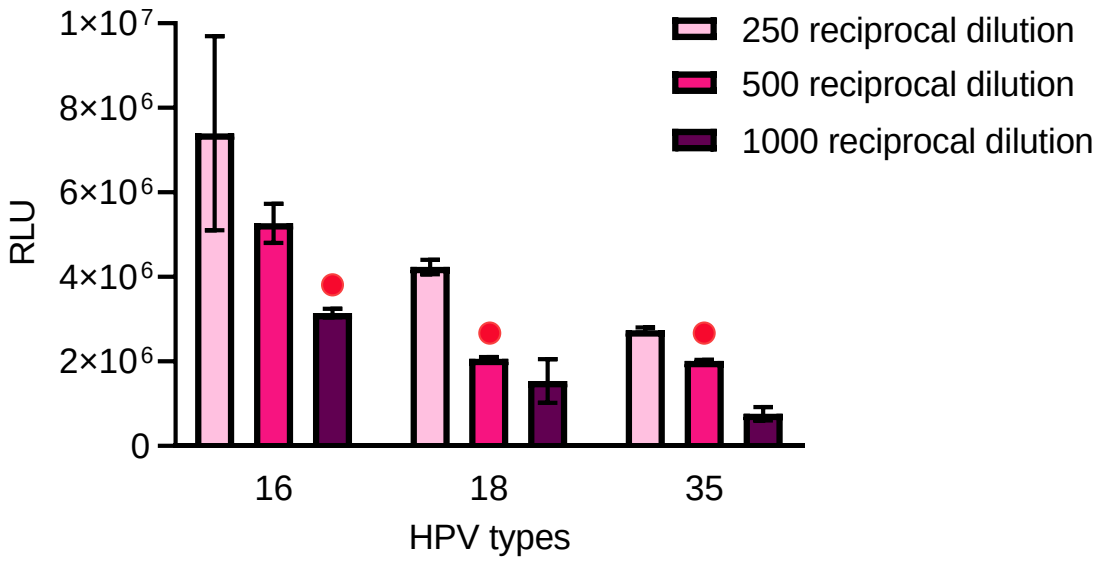


Figure 3.7: Selection of HPV PsVs dilution: PsVs were tested using 3 dilutions, to determine the minimum dilution of each HPV to be used in PBNAs. Error bars indicate standard deviation between duplicate readings.

Type-specific neutralizing antibodies by Christensen *et al.* (1996a) were used as positive controls and were tested at different dilutions (Table 3.3, Section 3.2.3.5.1), to achieve ~100% reduction of SEAP signals. All antibodies neutralized their corresponding HPV types and a suitable dilution was used as a control for each type (Table 3.5). HPV 6, 31, 45 and 52 PsVs were all used at 1:100 while HPV 58 PsVs were used at 1:500, meanwhile their corresponding NAbs were used at 1:20000 for H6.C6 and H31A6 (HPV 6 and 31, respectively), 1:100 for H52D11 and H45N5 (HPV 52 and 45, respectively) and 1:20 000 for H58J6.3 (HPV 58) (Megan Hendrikse, personal communication).

Table 3.5: Dilutions of positive neutralizing antibodies used in PBNAs

NAbs Name	HPV type	Dilution used in PBNAs	% neutralization
H16V5	HPV 16	1:5000	100
H18.J4	HPV 18	1:10000	95
H35Q8	HPV 35	1:500	99

3.3.2.4 Pseudovirion-based neutralization assays

Anti-L1 neutralizing antibodies were detected using PBNAs with NAbs as a positive control. Sera that resulted in 50% reduction of SEAP signals when compared to a PsVs only control were considered neutralizing positive and were further titrated to determine the endpoint of neutralizing titres. Plant-produced empty vector serum was used as a negative control for serum from mice immunized with plant-produced VLPs. Negative control serum was tested at the lowest dilution (1:4000) only.

Pooled sera were initially tested at 1:50 dilution to determine if there were any anti-L1 neutralization antibodies. All sera reduced type-specific SEAP signals by 100% (completely neutralizing) at 1:50 dilution (data not shown) and were further titrated. Sera were initially titrated using a 4-fold serial dilution ranging from 1:50-1:51200, however PsVs were completely neutralized up to a dilution of 1:12800 and endpoint titres could not be established from these data (data not shown). Based on these data, sera were re-titrated using a 4-fold serial dilution ranging from 1:4000 to 1:1024000.

Gardasil® serum

The Gardasil® vaccine has already been studied and shown to elicit homologous and heterologous neutralizing antibodies (Smith *et al.*, 2007a, Einstein *et al.*, 2009). Therefore, due to the high cost of the SEAP assay the neutralization ability of Gardasil® serum was only tested on HPV 16 and 18 PsVs in this study. Serum successfully neutralized both HPV 16 and HPV 18 PsVs, the cut off of anti-L1 neutralizing titres was a 50% reduction of SEAP signal (red-dashed line, Figure 3.8A). Serum neutralized PsVs at 4000-16000 titres by ~97%. The highest neutralization titre observed was 64000, which neutralized HPV 16 PsVs (<50% SEAP signal observed) but not HPV 18 PsVs (>50% SEAP signal detected) (Figure 3.8A). Suggesting that, the serum had more potent neutralizing antibodies for HPV 16 compared to HPV 18. This was not surprising as the vaccine contained a higher concentration of HPV 16 L1 than HPV 18 L1. However, no statistically significant difference in the neutralization ability between HPV 16 PsVs and HPV 18 PsVs, $p > 0.05$ ($p = 0.3191$), was observed. The positive neutralizing antibody controls successfully neutralized their corresponding PsVs, showing the validity of the assays (Figure 3.8B).

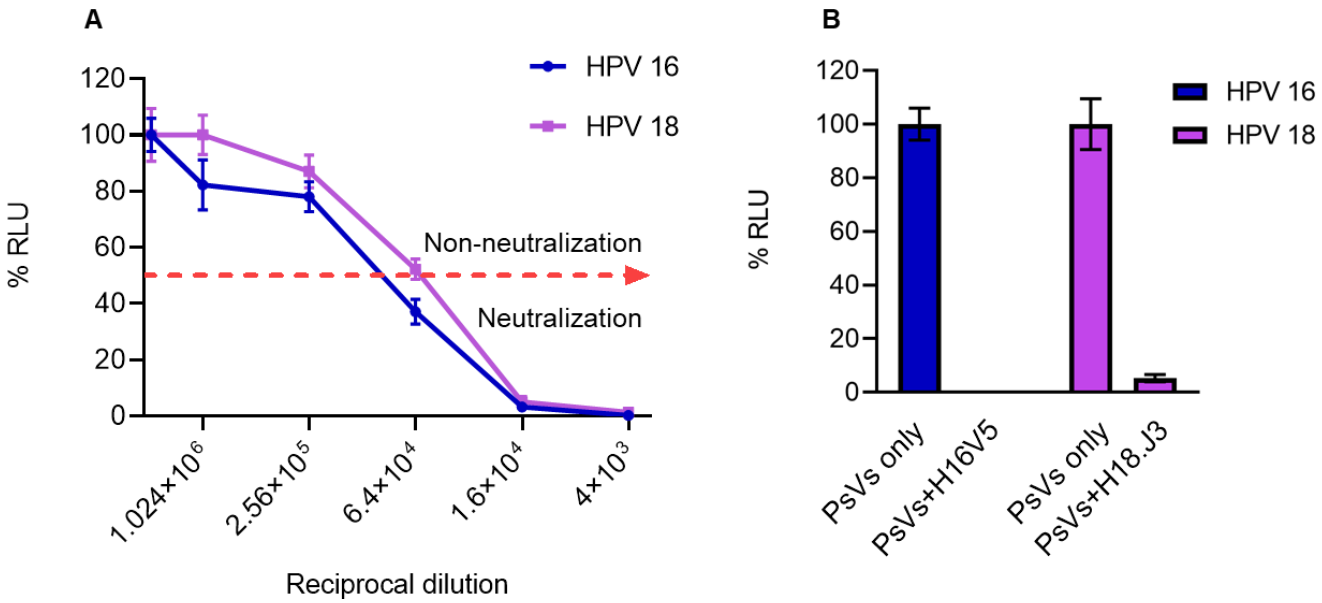


Figure 3.8: HPV 16 and 18 neutralization assays: A) Gardasil® serum B) PsVs and positive control antibodies (H16V5 for HPV 16 and H18.J3 for HPV 18). Markers and error bars indicate mean values and standard deviation from duplicate readings.

Serum from mice immunized with plant-produced VLPs

For plant-produced VLP serum, ~95% neutralization was observed at 1000-16000 titres for all the type-specific PsVs. Furthermore, ~91% neutralization was observed at titres of 64000 for all HPV types (Figure 3.9A). The highest neutralization titre of 256000 was observed for HPV 35 and 52 PsVs, 50% cut off (red-dashed line, Figure 3.9A). This indicated that serum from mice inoculated with plant-made VLPs had more potent neutralizing antibodies for HPV 35, followed by HPV 52 then HPV 58. However, there was no statistically significant difference in the neutralization ability between these types (p=0.1436). Empty vector sera did not neutralize any of the HPV PsVs, and the reduction in SEAP signal observed for HPV 35 and 52, might have been due to large variation between duplicate readings (3.9B). The ability of positive neutralizing antibody controls to successfully neutralize their corresponding PsVs, showed the validity of the neutralization assays (Figure 3.9B).

To my knowledge, there is no data on evidence of cross protection of heterologous HPVs by HPV 35, 52 or 58. Therefore, plant-produced VLPs sera, were tested against all the heterologous PsVs that were available in our lab (HPV 6, 16, 18, 31, and 45), to see whether they will neutralize any of them. However, plant-produced serum did

not neutralize any of heterologous PsVs tested in this study at 1:50 dilution of the sera (results not shown).

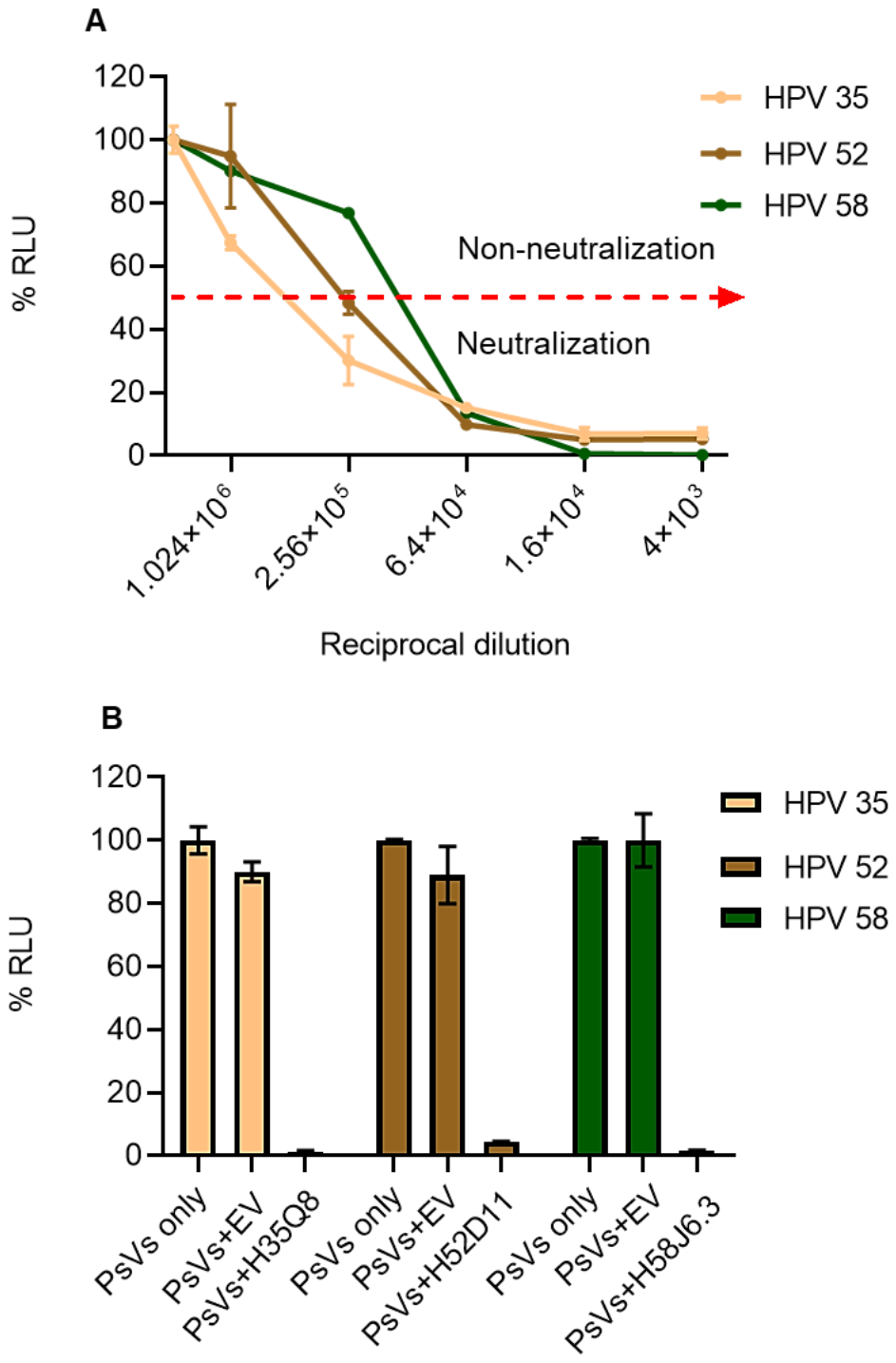


Figure 3.9: HPV 35, 52 and 58 neutralization assays: A) Plant VLPs serum B) PsVs; empty vector (EV) and positive control antibodies (H35Q8 for HPV 35 and H52D11 for HPV 52 and H58J6.3 for HPV 58). Markers and error bars indicate mean values and standard deviation from triplicate readings.

3.3.2.5 Overview of type-specific neutralizing anti-L1 titres of mice sera

Table 3.6 shows a summary of the neutralization ability of Gardasil® and plant-produced VLP sera obtained from immunized mice. Overall, all sera had type-specific neutralizing titres which successfully reduced homologous SEAP signals when compared to their corresponding PsVs only control. However, plant serum did not neutralize any tested heterologous PsVs.

Table 3.6: Neutralizing anti-L1 titres elicited by Gardasil and plant-derived VLPs.

HPV types	Vaccine group	Anti-L1 Neutralizing antibodies	~100% neutralization titre	~50% neutralization titre
16	Gardasil®	Y	16000	>64000
18	Gardasil®	Y	4000	64000
35	Plant-produced VLPs	Y	4000	>256000
52	Plant-produced VLPs	Y	4000	256000
58	Plant-produced VLPs	Y	16000	>64000
6	Plant-produced VLPs	N	-	-
31	Plant-produced VLPs	N	-	-
45	Plant-produced VLPs	N	-	-
35	Empty vector	N	-	-
52	Empty vector	N	-	-
58	Empty vector	N	-	-

Y= Yes; N= No; - = None

3.4 Discussion

3.4.1 Anti-L1 antibodies in mice sera

HPV VLPs are generally known to have comparable antigenicity characteristics to native virions. This is because of their repetitive surface-displayed epitopes, ability to activate B-cells and particulate nature, especially their size 40-60nm (Fifis *et al.*, 2004). Their size seems to be ideal for nanoparticles uptake by dendritic cells (Aljabali *et al.*, 2018, Fifis *et al.*, 2004). In this study, the humoral immune response of Gardasil® and plant-made HPV L1 VLPs was assessed in mice to determine if plant-produced VLPs are suitable to make HPV vaccines.

Gardasil® is known to elicit humoral anti-L1 antibodies against high-risk HPV 16 and 18 (2 main oncogenic HPVs that are responsible for 70% of all cervical cancers) and low-risk HPV 6 and 11 (mainly responsible for genital warts) (Handisurya *et al.*, 2010, Joura *et al.*, 2008). Interestingly, Gardasil® has been shown to offer additional protection against HPV 31 (closely related to HPV 16) (Brown *et al.*, 2009a). Here, Gardasil® induced type-specific significant immune responses with the highest antibody titre of 51200 observed for all HPV tested (Figure 3.1B). Titres obtained in this study are similar to titres obtained elsewhere, where sera from Gardasil® immunized immune-competent individuals showed 6400-102400 titres in ELISAs (Handisurya *et al.*, 2010).

Plant-produced L1 VLPs elicited type-specific anti-L1 immune responses, and no anti-L1 was detected in the empty vector serum in ELISAs (Figure 3.2). However, high absorbance readings were obtained when plant-produced antigens were detected with empty vector and plant-produced VLPs sera, suggesting that the serum contained antibodies against co-purified host cell proteins (results not shown). This was not unusual for the purification method used in this study and had been reported on before (Chabeda *et al.*, 2019). Furthermore, heparin chromatography purification of plant-produced L1:L2 chimeras or yeast-produced L1 also resulted in host cell proteins co-purification (Pineo *et al.*, 2013, Kim *et al.*, 2010). The commercially available HPV vaccines are expensive partially due to their purification procedures (Chen and Lai, 2013). Therefore, co-purification of host proteins is not unique to plants, and need to be addressed. Here, pre-absorption of serum against crude plant extract from non-

infiltrated plants resulted in the removal of plant antibodies, which was evidenced by decreased absorbance readings (Figure 3.2).

Plant-produced L1 antigens have been reported to induce type-specific humoral immune responses (Chabeda *et al.*, 2019, Fernández-San Millán *et al.*, 2008, Maclean *et al.*, 2007, Biemelt *et al.*, 2003, Varsani *et al.*, 2003, Warzecha *et al.*, 2003). The highest anti-L1 titre observed in serum from mice inoculated with 6µg of plant-produced L1 in this study was 12800 for all HPV included in the vaccine (Figure 3.2B). Chabeda *et al.* (2019) reported titres of 6400 after immunizing mice with 5µg of plant-produced HPV 16 L1 VLPs. The higher titre (12800) observed in the current study suggested the presence of more intact VLPs with better displayed epitopes, therefore better uptake by dendritic cells. Furthermore, mice in this study were hyper-immunized which might have further contributed to the higher titre observed. Plant-made HPV 16 L1 have been reported to induce higher titres than those reported in this study, titres-20000, 51200 and 40960 (Pineo *et al.*, 2013, Fernández-San Millán *et al.*, 2008, Maclean *et al.*, 2007). High anti-L1 titres in these studies might be associated with the amount of L1 VLPs (10-30µg) used to immunize mice.

Western blots showed the detection of type-specific HPV L1 proteins at ~56 kDa with Gardasil® and plant-produced VLPs sera, with no L1 bands observed in the pre-bleeds and empty vector control in case of plant serum (Figure 3.3 and 3.4). Western blots experiments were validated by the detection of the same bands by the positive rabbit-raised Gardasil® serum (Figure 3.3B and Figure 3.4D). However, non-specific bands at ~82 and 32 kDa with high background signals were observed in westerns detected with non-pre-absorbed serum, especially in the empty vector (Figure 3.4B). These results are consistent with results previously reported in our lab. Chabeda *et al.* (2019) observed background signals when detecting plant-made HPV 16 antigens on western blots with sera from mice vaccinated with plant-made HPV 16 L1 and L1/L2 chimaera VLPs. Furthermore, Pineo *et al.* (2013) reported non-specific bands at ~80 kDa after detection of *E. coli* made HPV 16 L2 antigens with sera from mice inoculated with plant extract. These illustrated the presence of host cell contaminants in the vaccine samples, further demonstrating the need to explore purification methods for HPV VLPs to achieve pure vaccine samples. In this study, antibodies against host cell proteins were reduced by pre-absorbing sera against plant proteins. Background signals were

low after using pre-absorbed sera, suggesting the specificity of anti-L1 antibodies after pre-absorption of the sera.

Non-pre-absorbed plant-produced VLPs serum were further analysed for their ability to detect type-specific mammalian-made L1 and to assess whether any background signal will be observed. As expected the serum successfully detected HPV L1 proteins at ~56 kDa with no non-specific bands and/or background observed (Figure 3.4C). This suggested that the background signals observed was specific for plant contaminants.

3.4.2 Neutralization assays

Potential prophylactic HPV vaccine candidates should be able to elicit neutralizing antibodies (Rybicki, 2010). Therefore, in this study, neutralization ability of anti-L1 from mice serum was analysed using PBNAs- the gold standard assessment of the efficacy of HPV vaccine candidates (Lamprecht *et al.*, 2016).

As described in Section 3.3.2, PsVs used in this study were expressed in mammalian cells due to interference with plant-made antigens observed in ELISAs and western blots (Section 3.3.1.1 and 3.3.1.2). However, mammalian expression system is expensive and might hinder the development of affordable HPV vaccines. Plant-produced HPV 16 PsVs are feasible and have been tested by our group (Lamprecht *et al.*, 2016). Testing sera on plant-made PsVs in PBNAs would have been a good idea, as it will reduce the cost associated with the production of HPV vaccines and needs to be explored. However, it was beyond the scope of this study to make all the PsVs tested in both plant and mammalian cells. Therefore, for the purpose of this study, mammalian-made PsVs were used in PBNAs, as no background signals were observed after the detection of mammalian-made L1 on western blot (Figure 3.4E).

Gardasil® serum neutralized HPV 16 and 18 PsVs at titres of 4000-64000 (Figure 3.8A). These titres are in range with the neutralizing titres observed from sera of mice immunized with 0.1x human dose (25600- 51200 titres) (Wu *et al.*, 2015). Furthermore, titres of 1600-25600 were obtained from humans immunized with Gardasil® (Handisurya *et al.*, 2010).

Serum from mice immunized with plant-produced L1 also elicited type-specific neutralizing antibodies. Neutralization was observed at 4000-64000 titres for HPV 58,

and 4000-256 000 titres for HPV 35 and 52 (Figure 3.9A). L1 neutralization titres observed in this study were ~40-fold higher than titres reported in other studies- using plant-produced HPV 16 VLPs against type-specific PsVs: 6400 (Chabeda *et al.*, 2019, Maclean *et al.*, 2007); 50-500 (Pineo *et al.*, 2013) and 400 (Fernández-San Millán *et al.*, 2008). However, mice were hyper-immunized in this study, which might explain the high titres observed here. The ability of sera to prevent cell infection by PsVs have been associated with defence against experimental and natural infections (Breitburd *et al.*, 1995). Therefore, plant-derived HPV VLPs have the potential to be used as prophylactic vaccine, which might alleviate cervical cancer and related diseases in resource-poor countries.

No neutralization activity was observed against heterologous HPV 6, 16, 18, 31, and 45 PsVs in this study. This however, was not surprising as there is no data on the evidence of cross-protection between these HPV types. Mice were immunized with the total L1 dose that was made up of VLPs with aggregates/capsomeres, and not with a homogenous population of VLPs. Therefore, there is a probability that the L1 neutralizing epitopes for the aggregates/capsomeres were not sufficiently displayed to create neutralizing antibodies against heterologous PsVs or these HPV types just do not cross-neutralize.

I cannot directly compare the immune response of plant-produced VLPs to that of Gardasil® for different reasons. Firstly, these VLPs were produced in different expression systems and have different adjuvants, and secondly different doses were used in this study. Gardasil® was used at 1/5th human dose (24µg) while plant-produced VLPs were used at 6µg. However, plant-produced VLPs induced humoral immune response effectively as the commercially available HPV Gardasil® vaccine. This was evidenced by the type-specific anti-L1 neutralizing titres observed (Figure 3.9), suggesting that plant-produced VLPs are suitable for making HPV vaccines.

In conclusion, hyper-immunizing mice with Gardasil and pooled plant-produced VLPs of HPV 35, 52 and 58 successfully induced type-specific humoral immune response with neutralizing antibodies. This work has successfully demonstrated that plant-made HPV VLPs are highly and appropriately immunogenic, demonstrating the potential to use plant-based transient expression systems to produce cost-effective HPV VLP-base vaccines, particularly for developing countries.

4 Chapter 4: Conclusions and future work.

Commercially available HPV vaccines are effective at preventing HPV infections. However, these vaccines are expensive, which is preventing their use in developing countries, where ~85% of cervical cancer deaths occur (Ferlay *et al.*, 2018). Consequently, there is a need to develop affordable HPV vaccines, which was of specific interest here. Plants have been utilised as a cost-effective alternative to conventionally used expression systems. Therefore, the aim of this study was to evaluate the plant-based transient expression system as a tool to develop cost-effective multivalent prophylactic HPV vaccines that can potentially be used in low-and-middle income countries. The highest burden of cervical cancer is reported in Africa (Zhai and Tumban, 2016), therefore high-risk HPV types that are relevant in an Africa context were of main interest in this study.

Human-codon optimized L1 of the 8 most common oncogenic HPV types in Africa (HPV 16, 18, 31, 33, 35, 45, 52 and 58) and 2 low risk HPV types (HPV 6 and 11) were transiently expressed in *N. bethamiana* using the pTRACTP plant expression vector, which targets proteins to the chloroplast. All 10 types were successfully expressed in plants and assembled into VLPs, with capsomeres and aggregates also observed in the same fractions.

Purification of VLPs by sucrose and Optiprep™ density gradient ultracentrifugation resulted into co-purification of host cell proteins in this study. Therefore, research focusing on improving the purity of plant-produced HPV VLPs, without compromising the yield and structural integrity of these VLPs need to be established. Several chromatography strategies such as heparin, cation-exchange and size exclusion have been explored for the purification of HPV VLPs, however host cell proteins were evident (Pineo *et al.*, 2013, Kim *et al.*, 2010). Additionally, purification of HPV VLPs by these methods is time consuming and may result in significant L1 losses and/or degradation (Park *et al.*, 2008). Commercially available HPV vaccines are made by *in vitro* disassembly/reassembly of purified L1 to get homologous population of VLPs (Zhao *et al.*, 2014). Therefore, optimizing disassembly/reassembly of plant-produced VLPs might overcome the presence of host cell proteins in the vaccines, and ultimately bridge the gap between the yield, structural integrity and purity of plant-made HPV VLPs.

In this study, mice were hyper-immunized with Gardasil® and plant-made VLPs. Gardasil® elicited a strong humoral immune response against the tested HPV 6, 16 and 18 antigens. HPV 11 could not be tested, as we do not have the HPV 11 L1 gene that can be expressed in plants to make L1 proteins. It is reported in literature that, Gardasil® can induce neutralizing antibodies against homologous (HPV 6, 11, 16 and 18) and heterologous (HPV 31) types. Therefore, for the purpose of this study, the neutralization ability of sera from mice immunized with Gardasil® was only tested on the 2 main oncogenes HPV 16 and 18. As expected, sera successfully neutralized HPV 16 and 18 PsVs.

Plant-produced VLPs selected for mice studies also elicited strong humoral immune responses with type-specific neutralizing antibodies. However, sera from mice immunized with plant-produced VLPs did not neutralize any of the heterologous HPV (-6, 16, 18, 31, and 45) PsVs tested, these results were not surprising as to my knowledge there is no data on evidence of cross-protection of tested heterologous types by HPV 35, 52 and 58. Mice were immunized with a mixture of VLPs, capsomeres and aggregates, and there is a possibility that the L1 neutralizing epitopes were not sufficiently displayed on the surface of capsomeres and aggregates to create neutralizing antibodies against heterologous PsVs or these HPV types do not cross-neutralize. In summary, *in vitro* disassembly/reassembly of plant-produced L1 to make homogenous preparations of VLPs should be investigated to improve the homogeneity of the vaccine preparation, this could potentially improve neutralizing antibody titres obtained after vaccination with the plant-made VLPs.

This study successfully demonstrated that plant-produced VLPs are highly immunogenic and are potentially suitable for making multivalent prophylactic HPV vaccines, especially for developing countries. This can be achieved by expressing different HPV types in plants, and ultimately pool them to make a single vaccine that helps protect against multiple HPV types like Gardasil9®. Multivalent vaccines help reduce the number of injections required as it covers multiple serotypes in one vaccine.

Previous research in our lab has successfully made HPV 16 PsVs in plants with comparable results to PsVs produced by mammalian cells (Lamprecht *et al.*, 2016). It would be therefore interesting to evaluate sera from mice vaccinated with plant-

made VLPs using plant-made PsVs to compare with the neutralization results obtained in this study. This will assess whether antibodies against native plant proteins had any effect on the neutralization assays performed in this study.

This study fits in with a broader aim that would be to produce anti-HPV single chain fragment variable (scFvs) antibodies from the spleens obtained from the hyper-immunized mice. Spleens obtained from this study will be used to create an HPV antibody library using the phage library approach. The library will be screened for binders (scFv) to HPV antigens after which the scFV sequences will be cloned into plant expression vectors to produce these HPV antibodies in plants. These anti-HPV antibodies can be used either in research or as diagnostic reagents, as there are no commercially available anti-HPV antibodies and diagnostic kits. CamVir is the only commercially available monoclonal antibody for detection of HPV 16. However, it cannot be used to detect other HPV types. Expression of antibodies in plants is a well-established method (for review see Yusibov *et al.* (2016)) and producing feasible anti-HPV antibodies in plants will allow their usage not only in developed countries but, also in developing countries.

In conclusion, this study successfully demonstrated that plant-produced VLPs can elicit strong humoral immune responses in mice, demonstrating the ability to use plant transient-expression system to develop affordable yet effective multivalent HPV prophylactic vaccines for use in developing countries.

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