Development and efficacy testing of plant-produced virus-like particle vaccines against H6 avian influenza virus in chickens

by

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Declaration

I, Tanja Smith (student number 17398518), hereby declare that this dissertation, “Development and efficacy testing of plant-produced virus-like particle vaccines against H6 avian influenza virus in chickens”, is submitted in accordance with the requirements for the Doctor of Philosophy (PhD) degree at University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

Tanja Smith

31 January 2020

Ethics statement

The author, whose name appears on the title page of this thesis, has obtained, for the research described in this work, the applicable research ethics approval. The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

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Summary

The South African poultry industry has been beset by sporadic H6N2 avian influenza infection (sub-lineage I and II) in chickens since the early 2000s, with economic losses resulting from reduced egg production and co-infection with other pathogens. An egg-based inactivated H6N2 vaccine (AVIVAC® AI; Deltamune (Pty) Ltd.) based on a 2002 sub-lineage I isolate is available, although substantial antigenic drift has occurred in H6N2 viruses since its implementation. Globally, seasonal and pandemic plant-produced hemagglutinin (HA)-based influenza virus-like particle (VLP) vaccines are in advanced clinical trials with proven efficacy, speed of production, cost-effectiveness, scalability and safety, although not yet established for poultry. In this study, H6 avian influenza VLPs (sub-lineage I and II, respectively) were transiently produced in *Nicotiana benthamiana* and tested for protective efficacy in the target host. A production platform has been established for H6 VLPs in *N. benthamiana* by optimising protein expression and purification to maximize yield and by assessing the feasibility of large-scale production and downstream processing in a preliminary study. Subsequently, the respective plant-produced H6 VLPs were formulated into vaccines and their capacity to reduce viral replication and shedding upon challenge with a 2016 H6N2 field isolate were established in specific-pathogen-free (SPF) chickens, in comparison to the commercial H6N2 vaccine. The plant-produced sub-lineage I VLP vaccine (768 HA units/dose) was highly immunogenic (mean hemagglutination inhibition (HI) titer 10.7 $\log_2$), reduced the oropharyngeal and cloacal viral shedding by more than 100- and 6-fold, respectively, and shortened the duration of oropharyngeal shedding by at least a week in comparison to the non-vaccinated control. Due to initial low yield of sub-lineage II VLPs, the maximum antigenic mass vaccine dose (48 HA units/dose)) resulted in substantially lower HA-specific antibody titers (mean HI titer > 4 $\log_2$), but still reduced viral shedding from the oropharynx by more than 5-fold in comparison to the non-vaccinated control. In contrast, the commercial vaccine not only failed to effectively reduce shedding in comparison to the non-vaccinated control, but exacerbated oropharyngeal shedding until day 21 after viral challenge, illustrating the antigenic dissimilarity between the commercial vaccine and a recent field virus. Plant-produced VLP vaccines, which facilitates differentiation between infected and vaccination animals (DIVA), presents a new generation of poultry vaccines that is highly efficacious and cost-effective with the major advantage of producing a tailored antigenically-matched vaccine candidate within a short space of time and holds enormous potential for the poultry industry.

List of abbreviations

%	Percentage
^{o}C	Degrees celsius
α	Alpha
Ω	Ohm
µF	Microfarad
µg	Micrograms
µl	Microliter
µM	Micromolar
A	Adenine
AI	Avian influenza
Ala (A)	Alanine
ANOVA	One-Way analysis of variance
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
BFAP	Bureau for Food and Agricultural Policy
BSA	Bovine serum albumin fraction
C	Cytosine
CDC	Centers for Disease Control
cGMP	Current Good Manufacturing Practise
Co^{2+}	Cobalt ions
CSIR	Council for Scientific and Industrial Research
Ct	Cycle threshold
Cu^{2+}	Copper ions
CV	Column volumes
DAFF	Department of Agriculture, Forestry and Fisheries
DARPA	Defence Advanced Research Projects Agency
DIVA	Differentiation between Infected and Vaccinated Animals
dpc	Days post challenge
dpi	Days post infiltration
DSP	Downstream Processing
DTT	Dithiotreitol
EDTA	Ethylenediamine tetra acetic acid
EID_{50}	Embryo infectious dose 50
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic Reticulum
et al.	*et alia* (and others)
FDA	Food and Drug Administration
Fe^{3+}	Iron ions
g	Grams
G	Guanine
Gal	Galactose
Gly (G)	Glycine
GMP	Good Manufacturing Practice
GMT	Geometric Mean Titer

GUS	Beta-glucuronidase
HA	Hemagglutinin
HA_0	The full-length HA protein
HA_1 and HA_2	The two subunits of HA
HAU	Hemagglutinating units
HI	Hemagglutination Inhibition
His	Histidine
HPAI	High Pathogenic Avian Influenza
HRP	Horseradish peroxidase
IDA	Iminodiacetic Acid
IMAC	Immobilized Metal ion Affinity Chromatography
IVPI	Intravenous Pathogenicity Index
K_2HPO_4	Dipotassium phosphate
KCl	Potassium chloride
kDa	Kilodaltons
kg	Kilogram
KH_2PO_4	Monopotassium phosphate
kV	Kilovolts
LB	Lysogeny broth
LC-MS/MS	Liquid chromatography-mass spectrometry-based peptide sequencing
LPAI	Low Pathogenic Avian Influenza
Lys (K)	Lysine
M1	Matrix protein 1
M2	Matrix protein 1
MES	2-N-morpholino-ethanesulfonic acid
mg	Milligrams
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
MW	Molecular weight
NA	Neuraminidase
NaCl	Sodium chloride
$NaHPO_4$	Sodium phosphate
NI	Neuraminidase inhibition
Ni^{2+}	Nickel ions
NLS	N-Lauroylsarcosine sodium salt
nm	Nanometre
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2 (also known as nuclear export protein (NEP)
OD	Optical density
OIE	World Organization for Animal Health
PA	Acidic polymerase protein
PB1	Basic polymerase protein 1
PB1-F2	Basic polymerase protein 1 frame 2

PB2	Basic polymerase protein 2
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pH	Potential hydrogen
pI	Isoelectric point
PVX	Potato virus X
qRT-PCR	Quantitative Reverse Transcription PCR
RNA	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse Transcription PCR
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S/N	Sample to Negative control ratio
SA	Sialic acid
SANAS	South African National Accreditation System
SAPA	South African Poultry Association
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
Ser (S)	Serine
SOC	Super Optimal broth with Catabolite repression (SOC)
SPF	Specific-pathogen-free
T	Thymine
Ta	Annealing temperature
TAE	A buffer containing Tris, Acetic acid and EDTA
TED	Tris-(carboxymethyl)-ethylenediamine
TEM	Transmission electron microscopy
TFF	Tangential flow filtration
TMV	Tobacco mosaic virus
Tris	Tris(hydroxymethyl)aminomethane
TSP	Total soluble protein
UP	University of Pretoria
V	Volts
Val (V)	Valine
VLP	Virus-like particle
vRNA	Viral RNA
vRNPs	Viral Ribonucleoprotein Complex
WHO	World Health Organization
w/v	Weight/volume
www	World wide web
YMB	Yeast Mannitol broth
Zn^{2+}	Zink ions

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Peer-review scientific publications and conference proceedings

The results of this thesis have been published in *Plant Biotechnology Journal* and presented at two local conferences, with further papers in preparation:

- Smith T., O'Kennedy M.M., Wandrag D.B.R., Adeyemi M., Abolnik C. (2019). Efficacy of a plant-produced virus-like particle vaccine in chickens challenged with influenza A H6N2 virus. *Plant Biotechnology Journal,* available at: https://onlinelibrary.wiley.com/doi/pdf/10.1111/pbi.13219.
- Smith T., O'Kennedy M.M., Wandrag D.B.R., Adeyemi M., Abolnik C. (2019). The efficacy of a plant-produced virus-like particle vaccine against H6 avian influenza in specific-pathogen-free chickens. The 17th annual congress of the Southern African Society for Veterinary Epidemiology and Preventative Medicine (SASVEPM), Umhlanga, Kwazulu-Natal, South Africa, 19-23 August 2019 (oral presentation).
- Smith T., O'Kennedy M.M., Wandrag D.B.R., Adeyemi M., Abolnik C. (2019). The efficacy of a plant-produced virus-like particle vaccine against H6 avian influenza in specific-pathogen-free chickens. World Veterinary Poultry Association: South Africa Branch Congress, Monate Game Lodge, Limpopo, South Africa, 13-15 November 2019 (oral presentation).

Chapter 1
Literature review

1.1. Introduction to avian influenza

Influenza is a highly infectious acute viral respiratory disease of humans that affects all countries and communities. Seasonal influenza, which is caused by type A and B influenza viruses, affects an estimated 1 billion individuals worldwide each year, of which 3 to 5 million cases are considered to be severe, resulting in an estimated 290,000 to 650,000 deaths (Iuliano *et al.,* 2018; World Health Organization (WHO), 2019a). The medical care costs and productivity loss resulting from seasonal influenza places a substantial burden on economies (Cassini *et al.,* 2017; Putri *et al.,* 2018). The impact of pandemic influenza, a rare but reoccurring event resulting from the emergence of a novel influenza virus that humans have no immunity against, is even more substantial (WHO, 2018). Influenza pandemics are caused by type A influenza viruses which, unlike type B viruses that only have humans as host, can infect humans and a wide range of animals (including swine and avian species) and consequently have zoonotic potential (WHO, 2018). Four influenza pandemic outbreaks have occurred in the past hundred years, resulting in significant morbidity and mortality (Saunders-Hastings and Krewski, 2016; WHO, 2019b). Considering the increased economic globalization, urbanization and ease of travel across the globe, the next pandemic, which is considered to be inevitable, could spread at an unprecedented rate and result in significant disruptions (WHO, 2019b). In March 2019 the WHO launched the Global Influenza Strategy 2019-2030, which is aimed at reducing the burden of seasonal influenza, minimizing the risk of zoonotic influenza and mitigating the impact of pandemic influenza (WHO, 2019b). Thus, influenza results in substantial morbidity and mortality in human populations worldwide and is considered to be a serious public health concern, especially type A influenza viruses.

Avian species are considered to be an important reservoir of type A influenza viruses and play an important role in the evolution of human influenza A viruses (Webster *et. al.,* 1992; Suarez, 2017). Avian influenza (AI) viruses, or type A influenza viruses with an avian species as origin host, primarily infect wild birds and poultry. Migratory waterfowl are typically immune to disease, although morbidity and mortality of wild birds have occurred for some avian influenza viruses (WHO, 2015; CDC, 2017, Kleyheeg *et al.,* 2017). Migratory waterfowl can rapidly spread the virus to wild birds and poultry in new areas, occasionally resulting in the establishment of a stable lineage in domestic poultry. AI outbreaks can have a substantial impact on the poultry industry, which is one of the fastest growing sub-sectors in agriculture globally and is critical to food security and nutrition, particularly in developing countries (Alexandratos and Bruinsma, 2012; Mottet and Tempio, 2017). The production

losses, cost of containment, as well as the trade restrictions and embargoes placed on infected areas place a great burden on the economy, which is especially problematic for small-scale producers and/or developing countries (Rushton *et al.,* 2005; Otte *et al.,* 2008; Ramos *et al.,* 2017). Furthermore, several outbreaks of AI ("bird flu") in humans have been reported in numerous countries around the world and proved to be fatal in some cases, with infection attributed to exposure to infected live or dead poultry or contaminated surfaces (WHO, 2018). Although sustained human-to-human transmission with AI has not been documented, a new AI strain that can infect and be transmitted efficiently between humans who harbour no immunity against it could potentially emerge, which could cause severe illness and possibly death on a regional or global scale (Suarez, 2017; WHO, 2018; WHO, 2019b). Thus, AI has a substantial impact on the economy and society and controlling this disease at the animal source is critical to minimize the risk to human health.

1.2. Influenza viruses

Influenza (flu) viruses belong to the *Orthomyxoviridae* family and have a segmented ribonucleic acid (RNA) genome (Suarez, 2017; WHO, 2018). There are three types of influenza viruses (type A, B and C) known to infect humans, with type A and B viruses being more closely related to each other than to type C viruses (Gammelin *et al.,* 1990; Krossøy *et al.,* 1999; WHO, 2018). Influenza A viruses can infect a wide range of mammalian and avian species, while influenza B and C viruses primarily affect humans (WHO, 2018). A fourth type of influenza virus (type D) has recently been proposed, which primarily infects cattle and swine and is not known to affect humans (Hause *et al.,* 2013; Collin *et al.,* 2015; WHO, 2018). Influenza A and B viruses are responsible for seasonal influenza epidemics, while only type A influenza viruses have pandemic potential due to its animal reservoir (WHO, 2018). Type A influenza viruses are the aetiological agents of AI and will, therefore, be the focus of this review.

1.3. Type A influenza

1.3.1. Morphology

Type A influenza virus virions are pleomorphic and roughly spherical, ranging from 80 to 120 nm in diameter (Figure 1.1) (Webster *et al.,* 1992; Fujiyoshi *et al.,* 1994). At the centre is the negative sense single-stranded RNA genome, which is comprised of eight segments that encode at least eleven proteins: basic polymerase protein 2 (PB2) (segment 1), basic polymerase proteins 1 (PB1) and PB1 frame 2 (PB1-F2) (segment 2), acidic polymerase protein (PA) (segment 3), hemagglutinin (HA) (segment 4), nucleoprotein (NP) (segment 5), neuraminidase (NA) (segment 6), matrix (M) proteins M1 and M2 (segment 7) and non-

structural (NS) proteins NS1 and NS2 (also referred to as nuclear export protein (NEP)) (segment 8) (Webster *et al.,* 1992; Wise *et al.,* 2009; Dou *et al.,* 2017; Suarez, 2017). Each RNA segment is assembled as an individual viral ribonucleoprotein complex (vRNPs) consisting of viral RNA bound by several copies of NP and a single copy of the heterotrimeric viral polymerase complex (PB2, PB1 and PA). The eight vRNPs are typically organized in a 1 + 7 configuration (a central segment surrounded by seven segments of different lengths) inside the virus through possible interactions with each other and M1, which forms a monolayer around the virion core (Noda *et al.,* 2006). The virion envelope is a lipid bilayer derived from the cell membrane of the host during the viral budding process (Webster *et al.,* 1992). The three transmembrane proteins HA, NA and M2 (ion channel), which exists as a trimer, tetramer and tetramer, respectively, are embedded in the lipid envelope. The major surface glycoproteins HA and NA are glycosylated and are visible as spikes on the surface of the lipid envelope, with HA being the most abundant (Webster *et al.,* 1992; Fujiyoshi *et al.,* 1994; Nayak *et al.,* 2004).

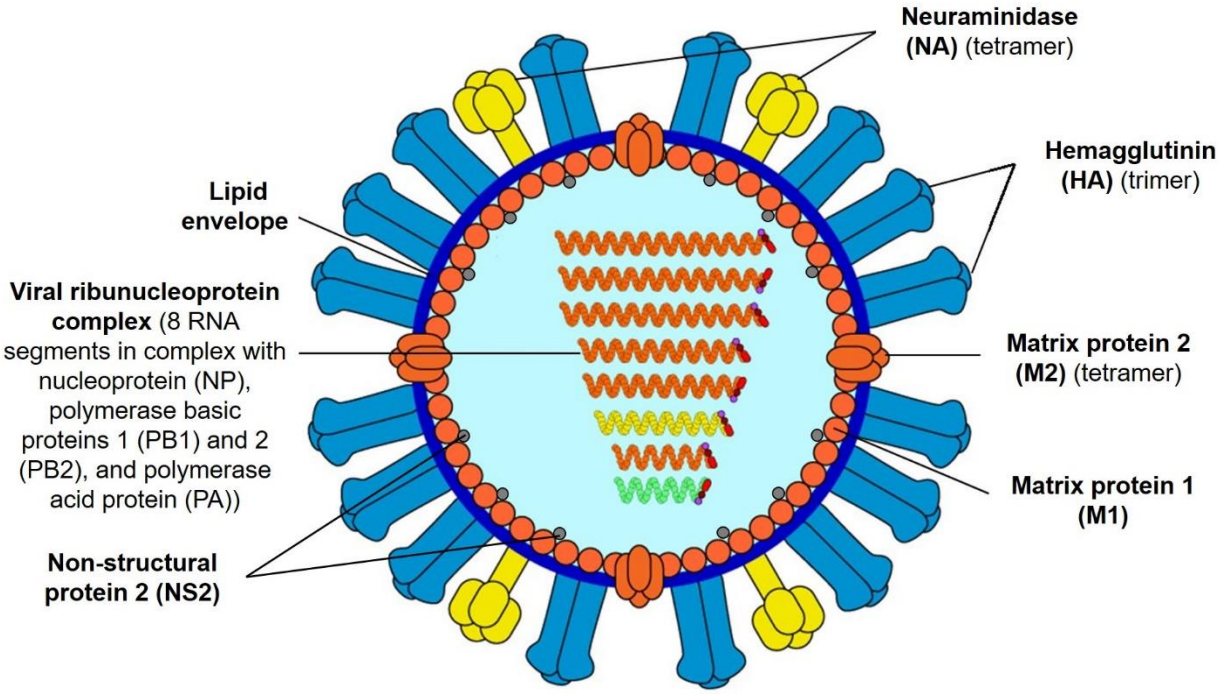


Figure 1.1: A schematic representation of the structure of influenza type A virus. Adapted from www.virologydownunder.blogspot.co.za/2016/02/.

1.3.2. Subtypes of type A influenza viruses

Type A influenza viruses are divided into distinct subtypes based on the genetic and antigenic properties of HA and NA, the surface glycoproteins responsible for viral entry and release, respectively (Webster *et al.,* 1992). Thus far, 16 HA subtypes (H1-H16) and 9 NA (N1-N9) subtypes that are not serologically cross-reactive have been identified in wild aquatic birds, with amino acid differences of between 20 and 63% for HA subtypes and between 31 and 61% for NA subtypes (Suarez, 2017). The HA subtypes can be divided into two groups: group 1 comprises subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16, while group 2 consists of subtypes H3, H4, H7, H10, H14 and H15. The NA subtypes can also be divided into two groups: Group 1 includes N1, N4, N5 and N8 subtype influenza A viruses, while group 2 comprises N2, N3, N6, N7, N9 (Swayne and Kapczynski, 2017). Additional HA (H17 and H18) and NA (N10 and N11) subtypes have been identified in bats (Wu *et al.,* 2014). Each influenza A virus has one HA and one NA antigen, in any combination.

1.3.3. Nomenclature

The nomenclature for describing influenza viruses have been standardized (WHO, 1980). The following features are included in the name of each isolate: 1) the type of influenza virus (e.g. influenza type A); 2) the host or origin, 3) the geographic origin of the isolate; 4) the unique reference identification number; 5) the year of isolation; and 6) the antigenic subtype, which are frequently included in parenthesis at the end (e.g. A/chicken/South Africa/H44954/2016 (H6N2). For human strains, the host is omitted.

1.3.4. Host range of type A influenza viruses

Type A influenza viruses can infect a broad range of species, including birds, humans, pigs, dogs, cats, seals and horses (Webster *et al.,* 1992; Suarez, 2017). All of the AI subtypes (H1-H16, N1-N9) have been identified in wild aquatic birds, typically without them showing signs of the disease, suggesting that they are the natural host species and biological reservoirs (Webster *et al.,* 1992; Röhm *et al.,* 1996). The virus is spread from infected birds to new susceptible hosts via saliva, nasal secretions or faeces, or through contact with contaminated surfaces (CDC, 2017). However, a restricted number of influenza subtypes has become established in mammals (including humans and pigs) and in domestic birds (poultry and captive birds), which is indicative of host range restrictions (Alexander, 2007; Brown, 2010; Suarez, 2017).

The major surface glycoprotein HA plays an important role in host range restrictions. The precursor polypeptide HA_0 (homotrimer) is cleaved extracellularly into two subunits (HA_1 and HA_2) linked by disulphide linkages, via host proteases (Skehel and Waterfield, 1975; Webster

et al., 1992; Cheung and Poon, 2007). The subsequent conformational change enables binding of viral particles (via HA_1) to sialic acid (SA)-containing receptors on the surface of host cells, as well as fusion between the virion envelope (via HA_2) and the host cell (Klenk *et al.,* 1975; White *et al.,* 1981; Webster *et al.,* 1992; Horimoto and Kawaoka, 1994). The amino acid sequence that makes up the HA receptor-binding site, which is located in the globular head domain of HA_1, determines if the influenza virus has a stronger affinity for SA-containing receptors linked to the galactose via an α2,3, or an α2,6 bond. The type of SA linkage affects the conformation of the host receptor protein and consequently affects virus binding. For example, for the H3 subtype, amino acids glutamine and glycine at codon 226 and 228, respectively, confer binding to the SA α2,3-Gal receptor (predominantly expressed in avian species and is referred to as the avian receptor), whereas leucine and serine at these positions confer binding to the SA α2,6-Gal receptor (referred to as the human receptor) (Connor *et al.,* 1994; Vines *et al.,* 1998). Glycosylation of the HA protein is essential for protein folding and transport to the cell surface and is also reported to play an important role in receptor binding activity (Tate *et al.,* 2014; Zhang *et al.,* 2015; Zhao *et al.,* 2015). Influenza A viruses have the ability to evolve rapidly, including alterations in glycosylation patterns, which could alter receptor preference and consequently the target host (Shi *et al.,* 2014).

The ability of the influenza viruses to adapt and evolve stems from the nature of its genome. Viral RNA polymerases lack proof-reading functions and influenza viruses are, therefore, prone to rapid mutations (Holland *et al.,* 1982; Webster *et al.,* 1992; Chen and Holmes, 2006). For avian influenza viruses, an estimated 1.8 to 1.84 x 10^{-3} nucleotide substitutions per site per year have been reported (Chen and Holmes, 2006). In addition, due to its segmented genome, type A influenza viruses are also prone to reassortment, the process by which co-infecting viruses exchange gene segments when two viruses happen to co-infect a cell (Webster *et al.,* 1992). Major genetic changes like genetic reassortment are referred to as antigenic shift, and occasionally lead to pandemics (WHO, 2019c). In contrast, minor genetic changes (antigenic drift), which include amino acid changes, occur continually over time and can result in repetitive influenza outbreaks (WHO, 2019c). Antigenic drift can also alter receptor-binding specificity of the influenza virus, which could result in a specific host or species that was previously impervious to the specific virus, to be susceptible to infection (Vines *et al.,* 1998; Shi *et al.,* 2014). Thus, due to the nature of its genome, type A influenza viruses are highly adaptable and able to spread quickly to new susceptible hosts, which is a major hurdle in the control of influenza A viruses.

1.3.5. Infection cycle of influenza A viruses

Influenza A viruses replicate inside the host cell and the infection cycle has recently been reviewed in detail by Dou and colleagues (2017). Viral infection is initiated when activated HA attaches to sialic acid-containing receptors (typically SA α2,3-Gal receptor or SA α2,6-Gal receptor) on the host cell (Webster *et al.,* 1992; Dou *et al.,* 2017; Suarez, 2017). The virus is endocytosed and the low pH in the endosome induces a conformational change in the HA protein, facilitating the fusion of the viral envelope and endosomal membrane via the fusion peptide localized in HA_2 (Webster *et al.,* 1992; Dou *et al.,* 2017; Suarez, 2017). Following acidification mediated by the M2 ion channel, vRNPs dissociate from M1 and are released into the host cytoplasm, whereafter they are actively transported into the nucleus for transcription and replication via the heterotrimeric polymerase complex and host proteins. Positive sense viral mRNA is transported to the cytoplasm to produce the viral protein components using the cellular machinery of the host, which is divided between cytosolic ribosomes (for the PB1, PB2, PA, NP, NS1, NS2 and M1 proteins) and endoplasmic reticulum (ER)-associated ribosomes (for the HA, NA and M2 proteins) (Dou *et al.,* 2017; Suarez, 2017). Synthesized viral proteins PB1, PB2, PA and NP (which forms the vRNPs with viral RNA) are imported into the nucleus, along with NS1, NS2 and M1 (Table 1.1) (Wang *et al.,* 2000; Shimizu *et al.,* 2010; Dou *et al.,* 2017). The PB1-frame 2 (F2) protein, which is the result of an alternative reading frame of the PB1 RNA segment, is transported to the mitochondria (Table 1.1) (Varga and Palese, 2011). The membrane proteins, namely HA, NA and small amounts of M2, are synthesized in the ER and modified in the Golgi apparatus, wherafter the mature forms are transported to the cell surface and integrated into the plasma membrane (Table 1.1) (Webster *et al.,* 1992; Dou *et al.,* 2017; Suarez, 2017). The NS2-M1-vRNPs complex and other M1 and NS2 proteins are transported out of the nucleus to the apical cell membrane and align beneath the integral membrane proteins for assembly into virions, which bud from the cell membrane. The release of progeny viral particles are mediated by NA, which removes sialic acid from the surface of the HA protein (Suarez, 2017). As mentioned previously, proteolytic cleavage of the HA_0 protein into its two sub-units HA_1 and HA_2 by host proteases is required for viral particles to be infectious. The HA protein can be activated already at the Golgi via ubiquitously expressed endoproteases like furin if polybasic amino acids are present at the HA_0 cleavage site, resulting in the release of infectious progeny viral particles from the host cell that can replicate in a number of different cell types (i.e. systemic infection). In contrast, if monobasic amino acids are present at the HA_0 cleavage site, the released progeny viral particles are non-infectious until the HA protein is cleaved, which occurs at the plasma membrane via serine proteases expressed in the epithelial cells of the respiratory tract (Suarez, 2017).

Table 1.1: The known genes and proteins of influenza A virus, along with the function and localization of the respective proteins after synthesis.

Gene segment	Gene	Protein	Proposed function	Localization of mature protein in infected host cell
1	*Basic polymerase protein 2 (PB2)*	PB2	Subunit of the heterotrimeric RNA-dependent RNA polymerase complex; involved in viral replication and expression	Nucleus
2	*Basic polymerase protein 1 (PB1)*	PB1	Subunit of the heterotrimeric RNA-dependent RNA polymerase complex; involved in viral replication and expression	Nucleus
		PB1-frame 2 (F2)	Implicated in virulence; induces apoptosis and inhibits host immune response (interferons)	Mitochondria
3	*Acidic polymerase protein (PA)*	PA	Subunit of the heterotrimeric RNA-dependent RNA polymerase complex; involved in viral replication and expression	Nucleus
4	*Hemagglutinin (HA)*	HA	Major glycoprotein; responsible for viral attachment and endosomal membrane fusion, primary target for neutralizing antibodies; implicated in virulence	Apical cell membrane
5	*Nucleoprotein (NP)*	NP	Encapsidates viral RNA; involved in RNA replication and the transport of viral components; a major target of the host cytotoxic T-cell immune response	Nucleus
6	*Neuraminidase (NA)*	NA	Glycoprotein (second most abundant); facilitates the release of progeny virus particles from the host cell	Apical cell membrane
7	*Matrix (M)*	M1	Forms a shell around the ribonucleoprotein complex and is the primary determinant of morphology; plays a role in nuclear export and progeny virus assembly	Nucleus and cytoplasm
		M2	Glycoprotein; ion channel – it allows acidify-cation during virus uncoating and regulates the pH in the Golgi during HA synthesis	Apical cell membrane
8	*Non-structural (NS)*	NS1	Not found in the progeny virus particles; involved in virus replication and downregulates dsRNA-induced antiviral responses; is implicated in virulence	Nucleus
		NS2	Primarily found in host cells but some protein is present in the virion; involved in nuclear export of vRNPs by binding to M1	Cytoplasm

1.4. Clinical disease of avian influenza viruses in poultry

Avian influenza viruses can be divided into two groups: those that cause systemic infection in terrestrial poultry (primarily chickens and turkeys) and those that result in mucosal infection in the respiratory tract and/or the gastrointestinal tract. Viruses that cause systemic infection and typically result in severe clinical signs and high mortality rates in chickens are referred to as high pathogenic avian influenza (HPAI) viruses and is restricted to H5 and H7 subtypes (Suarez, 2017; The World Organisation for Animal Health (OIE), 2018). Symptoms may include discoloration or swelling of various body parts, diarrhea, nasal and ocular discharge, coughing, snicking, incoordination, nervous signs, and a marked reduction in egg production in layer or breeder flocks (OIE, 2018). The HA_0 cleavage site is a major determinant of viral pathogenicity and is used in the classification of viruses as high or low pathogenic, at a molecular level. The presence of multiple basic amino acids (arginine and lysine) near the cleavage site is associated with high pathogenicity of influenza viruses, and glycosylation of the HA protein could also play a role in pathogenicity (Wood *et al.,* 1993; Horimoto and Kawaoka, 1994; Tate *et al.,* 2014; He *et al.,* 2015; Zhang *et al.,* 2015; OIE, 2018). HPAI viruses are not normally present in the wild bird host reservoir but instead arise following mutation(s) in terrestrial poultry, although HPAI viruses have been identified in wild birds (Röhm *et al.,* 1995; Suarez, 2017). The selection pressure for influenza A viruses to change into a highly pathogenic form is unknown at this time, although it is believed that the replication of these viruses in gallinaceous birds, including chickens, turkeys and ducks, play an important role in the process (Suarez, 2017). Viruses of the Guangdong HPAI H5Nx clade 2.3.4.4 lineage in particular, which have spread from East Asia to Europe, North America, West Asia and Africa since 2014, have resulted in substantial losses in wild birds, domestic poultry and zoo birds (Kleyheeg *et al.,* 2017; Lee *et al.,* 2017; Alarcon *et al.,* 2018). In South Africa, HPAI H5N8 viruses of clade 2.3.4.4 were identified in multiple wild bird and poultry species between June 2017 and May 2018, resulting in variable disease severity and increased mortalities in wild birds (Khomenko *et al.,* 2018). The 2017 H5N8 outbreak in commercial poultry in South Africa resulted in unparalleled losses to the poultry industry, with the total economic impact on broiler and laying sectors estimated to amount to R 1.87 billion (Bureau for Food and Agriculture Policy (BFAP), 2018). Due to the propensity of H5 and H7 subtypes to mutate and the potential to convert into highly pathogenic strains, all H5 and H7 outbreaks (of high and low pathogenicity) are required to be reported to regulatory authorities (OIE, 2018). Since 1959, more than 42 distinct epizootics of HPAI in domestic poultry and wild birds have been reported in various countries around the world, while the number of low pathogenic avian influenza (LPAI) H5/H7 outbreaks is unknown since these were not notifiable prior to 2006 (Swayne *et al.,* 2011*;* Swayne *et al.,* 2013; Lee *et al.,* 2017).

In contrast to HPAI, LPAI comprises all subtypes and typically causes a mild form of the disease in poultry (Suarez, 2017). LPAI infection typically results in increased mortalities, reduced egg production and mild to severe respiratory disease accompanied by increased secondary bacterial infections that require antibiotic treatment, although some infected poultry might be asymptomatic (Kinde *et al.,* 2003; Woolcock *et al.,* 2003; Suarez, 2017). However, the classification of a type A influenza virus as LPAI in chickens is not necessarily a predictor for the severity of the disease that could be expected in other host species (Suarez, 2017). There is also a significant risk that LPAI strains could contribute their replication competent genes to notifiable influenza strains (H5 and H7 subtypes) as a result of viral reassortment, or that the pathogenicity of a H5 or H7 LPAI strain could be converted to a HPAI strain as a result of mutation (i.e. insertion or substitution of basic amino acids at the HA_0 cleavage site) (Hoffmann *et al.,* 2000; Chin *et al.,* 2002; Gao *et al.,* 2013; Liu *et al.,* 2018; Suarez, 2017). Furthermore, human infection with LPAI viruses (including H6N1, H7N9, H9N2 and H10N8) that resulted in mild to severe forms of the disease have been reported, demonstrating that LPAI could also pose a threat to humans (Gao *et al.,* 2013; Wei *et al.,* 2013; Chen *et al.,* 2014). The H6 subtype in particular is considered to pose a potential threat to human health as H6 AI viruses have a broader host range compared to other subtypes, are frequently isolated from wild and domestic avian species around the world, are capable of forming stable lineages in poultry, and were able to infect different mammalian species with variable efficiency and transmissibility in several experimental studies (Abolnik *et al.,* 2007a, b; Munster *et al.,* 2007; Gillim-Ross *et al.,* 2008; Nam *et al.,* 2011; Zhang *et al.,* 2011; Wang *et al.,* 2014; Ni *et al.,* 2015; Wang *et al.,* 2015; Zou *et al.,* 2016; Ge *et al.,* 2017). Due to the potential economic and social impact of LPAI, the control and surveillance of LPAI viruses are of great importance.

1.5. Diagnosis of avian influenza viruses

1.5.1. Virus isolation

Virus isolation in embryonated chicken eggs is used primarily to diagnose the first clinical case in an outbreak and to obtain virus for further laboratory analysis (OIE, 2018). For virus isolation, samples collected from live or dead birds, including oropharyngeal and cloacal swabs, faeces, or samples of organs of dead birds, are suspended in antibiotic solution. The clarified suspension of each sample is inoculated into the allantoic cavity of at least five 9- to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs (or specific-antibody-negative eggs), followed by incubation at 37^{o}C for two to seven days (OIE, 2018). The allantoic fluid of each egg is subsequently recovered and tested for the presence of Influenza A viruses.

1.5.2. Virus detection and characterization

Influenza A virus can be identified by serology and/or molecular assays in accredited laboratories (OIE, 2018).

1.5.2.1. Hemagglutination, hemagglutination inhibition (HI), and neuraminidase inhibition (NI) assays

Hemagglutination and hemagglutination inhibition (HI) assays are based on the HA protein's ability to attach to red blood cells (erythrocytes) (Figure 1.2). For the hemagglutination assay, a two-fold serial dilution of the sample suspected to contain influenza A is tested for hemagglutinating activity, which is characteristic of influenza A viruses, avian avulaviruses, and a few strains of avian reoviruses (OIE, 2018). Erythrocytes not bound by influenza virus sink to the bottom of the microtiter plate well and form a distinct button, while erythrocytes attached to virus particles form a lattice that is held in suspension. The HA titer is calculated as the reciprocal of the dilution that shows complete agglutination of the chicken erythrocytes, which is referred to as one hemagglutinating unit (1 HAU). HA titers are considered to be positive if complete agglutination of chicken erythrocytes is observed at a sample dilution of 1/16 (2^4 or 4 $\log_2$ when expressed as the reciprocal) or more. Based on the HA titer, four hemagglutinating units (4 HAUs) of each positive sample is subjected to HI analysis to determine the presence of HA-specific antibodies in the sample. Antibodies to influenza A virus will prevent agglutination by binding to the virus and consequently inhibiting its attachment to erythrocytes (Figure 1.2). A two-fold serial dilution of the samples is tested against a positive antigen (an influenza A virus) and the end-point of the titration is the highest dilution wherein hemagglutination was completely inhibited (Figure 1.2). HI titers are considered to be positive if complete inhibition of hemagglutination was observed at a sample dilution of 1/16 (2^4 or 4 $\log_2$ when expressed as the reciprocal) or more. By making use of antisera to each of the HA subtypes the subtype of the isolated influenza A virus can be established. The neuraminidase inhibition assay, which is used for the identification of influenza A virus NA subtyping and/or the characterization of antibody in infected birds, is not as simple as the HI assay and has not gained widespread application. The NI assay is usually performed in an OIE Reference Laboratory. By making use of antisera to each of the NA subtypes, the subtype of the isolated influenza A virus can be established (Aymard-Henry *et al.,* 1973; OIE, 2018). Another serological test to identify NA-specific antibodies has also been described (Capua *et al.,* 2003). Although this indirect immunofluorescence antibody test that detects specific anti-N1 antibodies was found to be highly sensitive (98.1%) and specific (95.7%), it is considered to be time consuming, laborious and the interpretations of results could possibly be operator-subjective (Capua *et al.,* 2003).

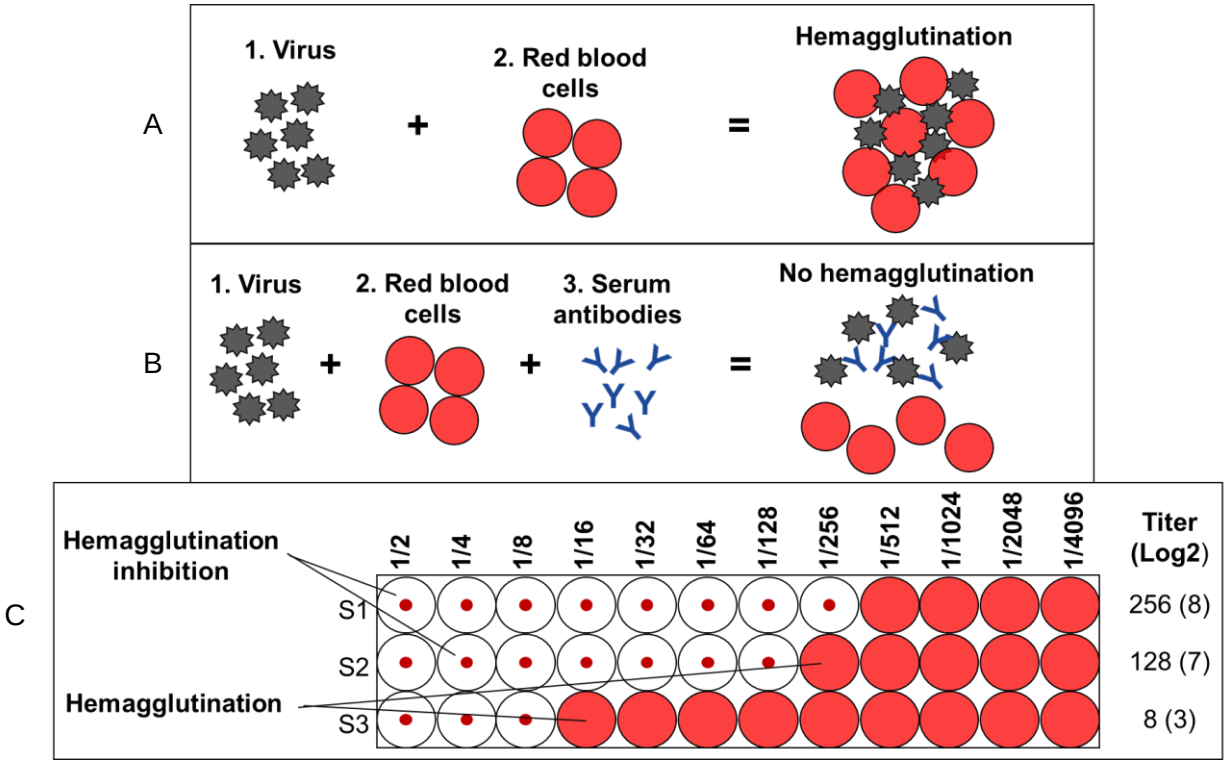


Figure 1.2. Schematic representation of the principle of the hemagglutination inhibition (HI) assay and an illustration of the HI assay for three samples in a microtiter plate. A) The hemagglutinin (HA) protein of the influenza A virus attaches to red blood cells and forms a lattice. B) However, in the presence of neutralizing antibodies, the antibodies will bind to the virus and prevent agglutination. C) The HI antibody titer is the reciprocal of the highest dilution against 4 HAU of antigen wherein hemagglutination is completely inhibited, which is visible by the formation of a distinct button (unbound red blood cells) at the bottom of the microtiter well: 256 (or 8 $\log_2$) for sample1 (S1), 128 (7 $\log_2$) for sample 2 (S2), and 8 (3 $\log_2$) for sample 3 (S3) (Adapted from https://microbeonline.com/hemagglutination-inhibition-test-hai-principle-procedure-result-interpretations/).

1.5.2.2. Enzyme-linked immunosorbent assays (ELISAs) for antibody detection

Validated commercial veterinary enzyme-linked immunosorbent assays (ELISAs) are available for the detection of influenza A virus-specific antibodies in poultry (OIE, 2018). These avian influenza virus antibody detection ELISA assays typically detects influenza A virus-specific antibodies by making use of a monoclonal antibody that targets an epitope on the nucleoprotein or matrix protein that is common to all influenza A viruses, as capture antigen. These antigen capture ELISA kits are of moderate cost and can be utilized for high-throughput screening for influenza A-specific antibodies, but might be restricted for use in specific species (OIE, 2018). For example, the BioChek Avian Influenza Disease Antibody Test kit (BioChek United Kingdom Ltd.) is used specifically as a screening tool for the

detection of influenza A antibodies in chickens and the type of antibody targeted is not specified, while the IDEXX Influenza A Ab test kit (IDEXX Laboratories, Inc., United States of America) is used for the detection of influenza A antibodies in multiple species (including avian and swine species) and target NP-specific antibodies. According to the OIE guidelines, all positive results need to be followed by HI testing for subtyping to H5 and H7. Some subtype-specific ELISA antibody test kits have also become available (e.g. H5, H7 and N1) (OIE, 2018).

1.5.2.3. Reverse transcription polymerase chain reaction (RT-PCR) for virus detection and characterization

Reverse-transcription polymerase chain reaction techniques (RT-PCR) typically targeting the *NP, M* or *HA* genes are considered to be a cheaper and more rapid alternative to virus isolation for detection of the virus (OIE, 2018). By making use of a fluorogenic hydrolysis probe to generate a target-specific fluorescence signal that can be monitored in real-time, rapid detection of influenza A virus in a closed system with sensitivity and specificity comparable to virus isolation can be attained (quantitative real-time reverse-transcription (qRT-PCR)) (Spackman *et al.,* 2003; OIE, 2018). RNA can be isolated directly from the clinical specimens of infected birds using standard extraction methods for analysis via RT-PCR. By making use of HA and NA subtype-specific primers (e.g. H5 or H7) real time RT-PCR assays and can be utilized for antigenic subtyping and determination of pathogenicity based on the HA_0 cleavage site (H5 and H7 subtypes), or sequence analysis of the HA and NA genes can also be performed (OIE, 2018).

1.5.3. Challenges with serology and molecular testing

Some influenza A viruses isolated from humans, swine, turkeys and chickens have lost the ability to agglutinate chicken erythrocytes due to antigenic drift, although the use of alternative red blood cells (e.g. turkey or guinea pig erythrocytes) proved to be effective (Rauff *et al.,* 2016; Suarez, 2017). A negative hemagglutination activity titer is typically indicative of a lack of viral growth in allantoic fluid. Failure to isolate influenza virus due to loss of agglutination activity using chicken erythrocytes could lead to a false negative result if a laboratory does not subsequently confirm the absence of influenza A virus via RT-PCR (Rauff *et al.,* 2016). Loss of agglutination activity would also hinder serological subtyping via HI, as well as molecular subtyping via RT-PCR or sequencing. If a circulating virus cannot be isolated to establish its genomic sequence, the oligonucleotide sequence of primers and probes cannot be updated as required, nor can phylogenetic analysis be performed to determine its similarity to other strains (Rauff *et al.,* 2016). Furthermore, for the subtyping of an influenza virus using HI, the most accurate results are obtained if the test antigen is

homologous or closely related to the virus being tested (Swayne *et al.,* 2015). Thus, diagnostic tests need to be routinely updated along with the evolving influenza A viruses to ensure accurate detection of circulating strains.

1.5.4. Pathogenicity testing

The intravenous pathogenicity index (IVPI) is used to determine the virulence of Influenza A viruses (OIE, 2018). The cultivated virus is injected intravenously into ten six-week-old SPF chickens, whereafter the birds are examined at 24-hour intervals for 10 days. Individual birds are scored on a scale of 0 to 3 based on the symptoms presented at each day of examination: 0 if normal, 1 if ill, 2 if severely ill and 3 if dead, with dead birds scored as 3 for each of the subsequent remaining daily observations. The IVPI score is the mean score per bird per observation over the 10-day period. An IVPI index of 3.0 means that all birds died within 24 hours, while an IVPI index of 0.0 means that no clinical signs were observed during the 10-day observation period. According to the criteria of the OIE, an H5 or H7 influenza A virus is considered to be highly pathogenic if an IVPI score greater than 1.2 is obtained in 6-week-old chickens, or if the amino acid sequence at the HA cleavage site has been demonstrated to be similar to other HPAI isolates via molecular techniques (OIE, 2018). The HA cleavage site is the best predictor of viral virulence in chickens, with the presence of multiple basic amino acids being associated with high pathogenicity (Suarez, 2017; OIE, 2018)

1.6. Vaccination as a tool to control influenza A virus in poultry

Vaccination against AI can be a very effective tool when used as part of a comprehensive control program, which should include strict biosecurity measures, educational programs, monitoring systems and depopulation following an outbreak (Swayne *et al.,* 2011; Swayne and Kapczynski, 2017). Vaccination against AI can be used preventatively, routinely, or in cases of emergency, and is aimed at reducing the morbidity and mortality of the disease and limiting the spread of infection (Swayne *et al.,* 2011). According to Swayne and colleagues (2011), 30% of countries affected by avian influenza between 2002 and 2010 (n=80) had made use of vaccination in the control of HPAI: 16% of countries vaccinated poultry (most often chickens, ducks and turkeys) exclusively, 10% vaccinated zoological and other birds, and 4% vaccinated both categories. In contrast, the usage of vaccines for the control of H5/H7 LPAI and other LPAI subtypes (primarily H9N2) were much lower (12% and 17%, respectively) and were only used in poultry (Swayne *et al.,* 2011). Between 2002 and 2010, more than 113 billion doses of AI vaccines were used to protect birds against HPAI in 15 countries, with China (91%), Egypt (4.6%), Indonesia (2.3%) and Vietnam (1.4%) accounting for the majority of usage. The total number of vaccine doses against H5/H7 LPAI (10.1 billion) was much less than that reported for HPAI, with Mexico (82.6%), Guatemala (8.9%),

El Salvador (6.3%) and Italy (2%) being the highest users (Swayne *et al.,* 2011). Persistent outbreaks of the H6 AI in poultry have resulted in several countries making use of vaccination against this LPAI subtype, including Taiwan, the United States of America and South Africa (Woolcock *et al.,* 2003; Rauff *et al.,* 2016; Wang *et al.,* 2016). As non-H5/H7 LPAI strains are not notifiable, the true extent of infection and the use of vaccination to aid in the control of AI is not known (Swayne and Kapczynski, 2017).

1.6.1. Immunological basis of protection of AI vaccines

Vaccination confers protection against clinical disease and/or viral shedding primarily through systemic humoral immunity, although cell-mediated immunity also reportedly plays a role (Swayne and Kapczynski, 2017). The HA protein is the major target for neutralizing antibodies and protection is consequently HA subtype-specific. Antigenic differences between HA subtypes, and between diverse strains within a specific HA subtype, have major implications for vaccination as it can significantly reduce the vaccine's effectiveness (Swayne *et al.,* 2006; Swayne and Kapczynski, 2017). Due to the high mutation rate of HA, particularly under vaccination pressure, it is recommended that vaccine strains should be re-evaluated every two to three years for efficacy against circulating field virus and updated if necessary (OIE, 2018). While vaccination reduces the risk of infection and the quantity of virus shed into the environment, and subsequently the spread of disease to susceptible hosts, absolute prevention of infection under most field conditions is not attainable (Swayne and Kapczynski, 2017).

1.6.2. Features of an ideal influenza vaccine

There are several factors that need to be taken into account in the development of an influenza vaccine for poultry. The ideal vaccine needs to be cost-effective, usable in multiple avian species, effective following a single vaccination (as opposed to prime-boost vaccination or multiple vaccinations), easily administered by mass application to large populations of poultry, allow differentiation between naturally infected and vaccinated animals (DIVA), able to overcome maternal antibody block (which inhibits the primary immune response), can be administered at 1 day of age or *in ovo*, and, very importantly, needs to antigenically match the field strain for optimal protection (Swayne and Kapczynski, 2017). Additionally, an influenza vaccine that can be produced rapidly and easily updated to antigenically match the latest field virus is highly advantageous. None of the licensed AI vaccines currently on the market meet all of the requirements for an ideal vaccine, although the use of a less-than-ideal vaccine can be advantageous over no vaccine use (Swayne and Kapczynski, 2017).

1.6.3. Assessment of AI vaccines for efficacy

The gold standard for assessing the efficacy of an AI vaccine is the use of LPAI and HPAI challenge models in the target poultry or surrogate avian species (Swayne and Kapczynski, 2017). Chickens are typically used in these challenge studies due to their popularity in poultry production and the fact that they are amenable to infection by both high and low pathogenic strains, resulting in the excretion of large quantities of virus that facilitates the study of viral transmission between animals and between facilities. In these studies, a group of birds vaccinated with a placebo and challenged with the specific influenza isolate is included as a control (Swayne and Kapczynski, 2017). The criteria used for determining efficacy in HPAI challenge include the prevention of morbidity and mortality, as well as the prevention or reduction of challenge virus replication and shedding from the oropharyngeal and gastrointestinal tracts. For LPAI challenge models, however, clinical signs (or death) are not typically produced and the main determinant of efficacy is a significant reduction (or at least a 100-fold reduction) in viral shedding titer, as well as the shortened duration of viral shedding, in comparison to the control group (Swayne and Kapczynski, 2017; OIE, 2018). The reduction in shedding can be assessed using qRT-PCR, while ELISA can be utilized to assess for example NP-specific antibodies, which can be used as a measure of challenge virus replication. The HI assay is typically used to measure HA-specific antibodies pre- and post-viral challenge, and pre-challenge HI titers have been used as a predictor of protection (Swayne *et al.,* 2015; Swayne and Kapczynski, 2017). In experimental studies involving SPF chickens and HPAI challenge strains that closely match the vaccine virus, HI antibody geometric mean titer (GMT) of ≥ 10 , ≥ 40, and ≥ 128 have been associated with protection against mortality, prevention of oropharyngeal viral shedding of most vaccinated birds, and complete protection against oropharyngeal shedding in all vaccinated chickens, respectively. However, apart from antigenic mismatch between the HA protein of vaccine and challenge strains, there are several factors that can affect the efficacy of an AI vaccine in experimental studies (Swayne and Kapczynski, 2017). These factors include 1) the challenge dose (with 10^6 mean embryo infectious dose (EID_{50}) being the most consistent challenge dose); 2) the quantity of HA in the vaccine (measured as EID_{50} or hemagglutinating units (HAU)/dose); 3) the choice of adjuvant (e.g. mineral oil) to enhance the immune response; 4) the route of administration of the vaccine and challenge material; 5) the number of vaccinations (with prime-boost being typical); 6) the species of bird; and 7) the age of the birds at vaccination. Thus, experimental design is critical and the prediction of protection is not absolute.

1.6.4. Types of avian influenza vaccines

1.6.4.1. Egg-based whole inactivated virus AI vaccines

The vast majority of registered AI vaccines used in the field, typically in the commercial poultry sector, are whole inactivated virus vaccines of the H5, H7 and H9 subtypes that are formulated with oil emulsion adjuvants and administered subcutaneously or intramuscularly (Swayne and Kapczynski, 2017). The oil emulsion enhances the immunogenicity of the vaccine by augmenting antibody production and extending the release of the antigen from the injection site. Between 2002 and 2010, more than 108 billion doses of licensed commercial oil-emulsified whole inactivated virus AI vaccines (which constitutes 95.5% of the total of 113.9 billion doses of H5 and H7 AI vaccines) were used in 15 countries to protect poultry against HPAI, the majority of which (91%) were used by China, the world's largest poultry producer and consumer (Swayne *et al.,* 2011). During this same period, a much smaller number of vaccine doses (10.1 billion) were used in poultry against H5/H7 LPAI, of which 57% (5.76 billion) were oil-emulsified whole inactivated virus AI vaccines (Swayne *et al.,* 2011). Most inactivated vaccine seed strains are of the low pathogenic type selected from field outbreaks and provides effective protection against both LPAI and HPAI. However, some countries have made use of HPAI virus vaccine seed strains for inactivated vaccines to assist in epidemics since 1995 (Swayne *et al.,* 2011; Swayne and Kapczynski, 2017). Due to fewer biosafety and biosecurity concerns, the use of LPAI strains for the production of AI vaccines is preferable.

Whole inactivated virus vaccines are predominantly produced by egg-based production methods. Using reverse genetics, a hybrid virus that contains the six internal genes of an influenza A vaccine strain (e.g. PR8) and the HA and NA genes of the field virus (or those of a closely related virus) is produced, thereby ensuring that the virus is able to replicate to high virus titers in fertilized SPF chicken eggs (Gerdil, 2003; WHO, 2009; Soema *et al.,* 2015; Swayne and Kapczynski, 2017). Alternatively, the NA gene of a virus not involved in the outbreak can be selected, thereby facilitating the differentiation between infected and vaccinated birds within the specific population (DIVA) (Capua *et al.,* 2003). After the virions are harvested from the allantoic fluid and chemically inactivated, the outer proteins of the virus are purified and tested for potency (Gerdil, 2003; WHO, 2009). Although egg-based vaccine production is well-established, it takes approximately three months before the purified, inactivated antigen is available in bulk, with a total production time estimated at five to six months (WHO, 2009). Additional drawbacks of egg-based vaccine production include the need for a high containment laboratory and the dependence on the supply of SPF eggs, of which the latter could be problematic during an outbreak of avian influenza in commercial flocks (Gerdil, 2003; Soema *et al.,* 2015). As a result, alternative non-egg-based production

methods have been explored for avian influenza vaccines (Table 1.2) (Swayne and Kapczynski, 2017).

Table 1.2: Advantages and disadvantages of different types of avian influenza vaccines, as discussed by Swayne and Kapczynski (2017).

Vaccine type	Advantages	Disadvantages
Whole inactivated egg-produced oil-emulsion vaccines	✓ Broad subhomotypic protection (primarily humoral immunity) ✓ Reverse genetic technologies can generate a virus with high growth capacity in embryonated chicken eggs, with a low pathogenic HA proteolytic cleavage site ✓ Prevents contact transmission	- Long production time (up to 6 months) - Maternal antibodies delay serological response (vaccination after 4 weeks of age) - Large quantities of fertile SPF eggs required - Introduction of egg-adaptive mutations - Non-vaccinated sentinel birds used as part of DIVA strategy
***In vitro* expressed viral proteins (e.g. insect cell cultures)**	✓ Broad interclade protection possible ✓ Short production time ✓ Scalable ✓ Potential for *in ovo* and mucosal vaccination for some systems ✓ DIVA compatible	- Presence of contaminating protein(s) (e.g. baculovirus for the baculovirus vector-insect cell expression system) - Non-mammalian glycosylation
Live avian influenza virus vaccines (*in vivo* expressed)	✓ Good protection (humoral and cellular immunity) ✓ Rapid protection (vs. inactivated vaccines) ✓ Mass immunization potential ✓ Attenuation of virulence using reverse genetic technologies ✓ DIVA compatible	- Potential production losses due to respiratory disease or drops in egg production - Virus can easily spread through transmission - Potential for mutation, reassortment, reversion to virulence
Vectored avian influenza vaccines (*in vivo* expressed)	✓ Broad subhomotypic protection (humoral and cellular immunity) ✓ Some vector systems can prevent contact transmission ✓ Application in hatchery (1 day of age) possible for some vectors ✓ Mass immunization potential for some vector systems ✓ DIVA compatible	- Inhibition of vector replication if active or passive immunity against the vector or insert (e.g. HA) is present
DNA vaccines	✓ More effective protection against antigenic variant influenza A viruses than inactivated vaccines	- Protection is not consistent - More vaccinations are typically required - Large quantities of expensive nucleic acids per dose required for effective protection

1.6.4.2. Virus-like particles (VLP) as alternatives to whole inactivated virus AI vaccines

One alternative to traditional egg-based influenza vaccine production is the use of virus-like particles (VLPs). VLPs are self-assembled protein structures that closely resemble the organization and conformation of native viruses but do not contain any core genetic material, making them non-infectious (Noad and Roy, 2003). VLP-based vaccines offer superior immunogenicity (both cellular and humoral immune responses) and antigen stability compared to other subunit vaccines, and are considered to be safe in comparison to attenuated or inactivated vaccines since they can be manufactured using recombinant technology (Noad and Roy, 2003; Bright *et al.,* 2007; Quan *et al.,* 2007; Chen and Lai, 2013). Recombinant VLP-based vaccines are also DIVA compliant due to the absence of internal viral proteins, which enables differentiation between the field strain and the vaccination strain in target animals. Insect cell-produced VLP vaccines against human papillomavirus (Gardisil®, Merck & Co.; Cervarix®, GlaxoSmithKline) and hepatitis B (Recombivax-HB®, Merck & Co.; Engerix-B®, GlaxoSmithKline) are currently on the market (Roldao *et al.,* 2010). However, as the cost per dose of a veterinary vaccine (for chickens in particular) is required to be considerably lower in comparison to that of a human vaccine, a low-cost expression platform like plants (which is described in section 1.8) is imperative for the production of an influenza vaccine for poultry.

1.7. Avian influenza in chickens in South Africa

The influenza A virus subtype H6N2 has been detected in chickens in South Africa since the early 2000s. It is suspected that the H6N2 subtype in South Africa originated due to genetic reassortment between H9N2 and H6N8 strains in ostriches (Abolnik *et al.,* 2007a; Abolnik *et al.,* 2007b). Abolnik and colleagues (2007a, b) demonstrated that two distinct lineages (H6N2 sub-lineage I and II) were present in chickens from the onset and phylogenetic analysis revealed that the sub-lineage derived from a common ancestor. The H6N2 infection in chickens, which started in 2002, appears to have become endemic in the country and production losses of up to 40% have been reported for affected farms (Abolnik *et al.,* 2007a). In South Africa the poultry industry is the largest contributor to the agricultural sector and "feeds the nation" and the impact of H6N2 outbreaks in commercial flocks on the economy is consequently of great importance (South African Poultry Association (SAPA), 2015).

Following the H6N2 outbreak in chickens in South Africa, a whole inactivated virus influenza vaccine was produced and commercialized to protect commercial flocks. This egg-produced H6N2 vaccine (AVIVAC® AI; Deltamune (Pty) Ltd., South Africa) was derived from a sub-lineage I virus isolated in 2002 and was still in use at the time of writing (Rauff *et al.,* 2016). Most, but not all flocks of broiler and layer breeders and laying hens are likely to be

vaccinated against H6 AI, with each bird receiving at least two or three vaccinations during the course of its lifetime. However, influenza A viruses have a high mutation rate, especially under vaccination pressure. Following more than a decade of vaccination, H6N2 viruses isolated from chickens in South Africa (2002-2019) have undergone substantial antigenic drift and antigenic diversity, especially sub-lineage I (Rauff *et al.,* 2016; Abolnik *et al.,* 2019). Sub-lineage I exhibited a noticeable higher mutation rate in comparison to sub-lineage II (7.7×10^{-3} and 4.05×10^{-3} nucleotide substitutions per site per year, respectively) for 2002-2012 isolates, as well as a three-fold increase in the emergence of potential antigenic sites (Rauff *et al.,* 2016). In addition, several viruses isolated in 2012 and 2013 have lost the ability to agglutinate chicken erythrocytes, which correlated with a pattern of predicted O-glycosylation sites at residues within the receptor binding domain (134, 137 and 141) (Rauff *et al.,* 2016). The two sub-lineages are diverging, as indicated by a decrease in amino acid identity between the sub-lineages from 2002 (94.5-94.9%) to 2012 (86.6-88.4%), and no sub-lineage II viruses have been isolated since 2015 (Rauff *et al.,* 2016; Abolnik *et al.,* 2019). For sub-lineage I viruses isolated from 2002 to 2019, sequence homology in the HA protein between the 2002 virus group and the 2012/2013 and 2015/2016 virus groups were 96.1% and 95.6%, respectively, and decreased to 90.5% between the 2002 virus group and a single virus from 2019 (Abolnik *et al.,* 2019). Furthermore, several mutations in HA, NA and PB2 have been identified in one or more of the H6N2 isolates analyzed, including mutations that have previously been associated with virulence in mammalian species and/or switching to human HA receptor binding preference (Table 1.3) (Abolnik *et al.,* 2019). Human-associated markers and other mutations in the PB1, PB1-F2, PA, NP, M2, NS1 and NS2 proteins that have previously been associated with airborne transmission, virulence and antiviral responses, were absent in the H6N2 isolates analyzed. However, altered N- and O-glycosylation patterns of the surface glycoproteins, which could also affect virulence and erythrocyte binding, have been identified (Abolnik *et al.,* 2019). At the time of writing, the impact of these mutations on the efficacy (i.e. a significant reduction in viral shedding) of the commercial H6N2 vaccine against circulating field strains has not been determined in a clinical study, nor have any subsequent field isolates been developed as a replacement vaccine seed strain.

Table 1.3: Mutations in South African H6N2 isolates (2002-2019) identified by Abolnik *et al.* (2019) that have previously been associated with virulence in mammalian species and/or hemagglutinin receptor binding type preference.

Protein	Mutation	Proposed function	Identification in South African H6N2 isolates
Hemagglutinin (HA)	Ala138Ser	Key mutations near the receptor binding site implicated in human receptor binding preference (instead of avian-type receptor binding) (Ni *et al.,* 2015; Qu *et al.,* 2017; de Vries *et al.,* 2017)	Identified in all sub-lineage I viruses since 2002, except for two isolates that contained the A138M mutation
	Val187Asp		Identified in sub-lineage I and II viruses since 2002
	Ala193Asn		Identified in all sub-lineage I strains since 2002, identified in one sub-lineage II 2002 isolate
Basic polymerase protein 2 (PB2)	Asp701Asn	Associated with high pathogenicity in mice (Li *et al.,* 2005)	Identified in one early sub-lineage I strain
	Lys702Arg	Associated with the capacity of avian influenza viruses to infect humans (Finkelstein *et al.,* 2007)	Present in early sub-lineage I and II 2002 viruses; identified in all but three sub-lineage I viruses since 2015

Ala: Alanine, Arg: Arginine; Asn: Asparagine; Asp: Aspartic acid; Lys: Lysine; Ser: Serine; Val: Valine

1.8. Plants as platform for the production of influenza VLP vaccines

Plant molecular farming (Biopharming), or the production of pharmaceutically important and commercially valuable compounds in plants and plant cell cultures, has become one of the major applications in applied biotechnology in recent years (Rybicki, 2014; Park and Wi, 2016; Morris *et al.,* 2017). The first product was produced about 30 years ago in *Nicotiana benthamiana*, a close relative of tobacco, and production has subsequently expanded to include strawberries, spinach, lettuce and duckweed (Morris *et al.,* 2017). Plant-based systems are highly scalable and economical, is considered to be safer to the public due to the reduced risk of transmitting a human or animal pathogen to humans, and allow for glycosylation of the target protein, which is reported to be essential for proper immunogenicity and stability of VLP antigens (D'Aoust *et al.*, 2010; Kim *et al.,* 2014; Kolotilin *et al.,* 2014; Walwyn *et al.,* 2015; Nandi *et al.,* 2016). Plant-based expression can entail either stable genetic transformation or transient infection with viral vectors, of which transient plant-based expression is usually employed due to a reduction in development time and production time

(Sainsbury and Lomonossoff, 2008). Given its short production time, the Defence Advanced Research Projects Agency (DARPA) considers transient plant-based production systems to be the best manufacturing platform to combat bioterrorism and pandemics (Morris *et al.,* 2017). Transient plant-based expression has been widely adopted for the production of pharmaceutically important and commercially valuable proteins like antibodies, enzymes, hormones and vaccine antigens, including VLPs (Chen, 2008; D'Aoust *et al.,* 2008; Landry *et al.,* 2010; Shoji *et al.,* 2011; Rybicki, 2014; Shoji *et al.,* 2015; Sack *et al.,* 2015). For the purpose of this study, the focus will be on transient plant-based expression.

1.8.1. Transient plant-based expression using agroinfiltration

Transient plant-based expression makes use of vectors based on RNA plant viruses and *Agrobacterium*-mediated delivery of the viral vector containing the foreign gene into plant cells for recombinant protein expression (Sainsbury and Lomonossoff, 2008). *Agrobacterium tumefaciens,* an oncogenic soil pathogen that causes tumour formation in plants, have been modified ("disarmed") for its use as a delivery vehicle in transient plant-based expression without the formation of tumour-like growths (Hellens *et al.,* 2000). Agroinfiltration entails the introduction of the plant expression vector into the extracellular spaces in plant leaf tissue using a syringe without a needle (for laboratory-scale experiments) (Figure 1.3) or under a vacuum (for large-scale production). Thereafter, *A. tumefaciens* delivers the plant expression vector into the plant cell and a discrete portion of its genome that contains the gene of interest is transported to the nucleus for transcription via external viral replicases. *N. benthamiana* is one of numerous plant species harnessed for the transient production of pharmaceutically and commercially valuable molecules as it is susceptible to a variety of plant viruses, which facilitates replication of the *Agrobacterium*-mediated delivered gene(s) incorporated into the selected plant-expression vector (Goodin *et al.,* 2008). The development of a stable *N. benthamiana* line that facilitates mammalian-like glycosylation, which is of importance for the production of therapeutically relevant recombinant proteins for human health as the absence of these plant-specific glycans could eliminate potential negative side-effects, has contributed to its popularity as host (Strasser *et al.,* 2008). Agroinfiltration is a simple method that is particularly well suited for high-throughput studies and usually allows the recovery of high yields of protein in a short time frame (typically 3-7 days) (Schöb *et al.,* 1997; Goodin *et al.,* 2008; Shamloul *et al.,* 2014). There are, however, various factors at all the stages of the agroinfiltration process (Figure 1.3) that can influence protein expression levels and production time, and no combination of variables is equally effective for the expression of all heterologous protein. Thus, transient plant-based expression using agroinfiltration is a very effective method to produce recombinant protein, although optimization is required to maximize yield.

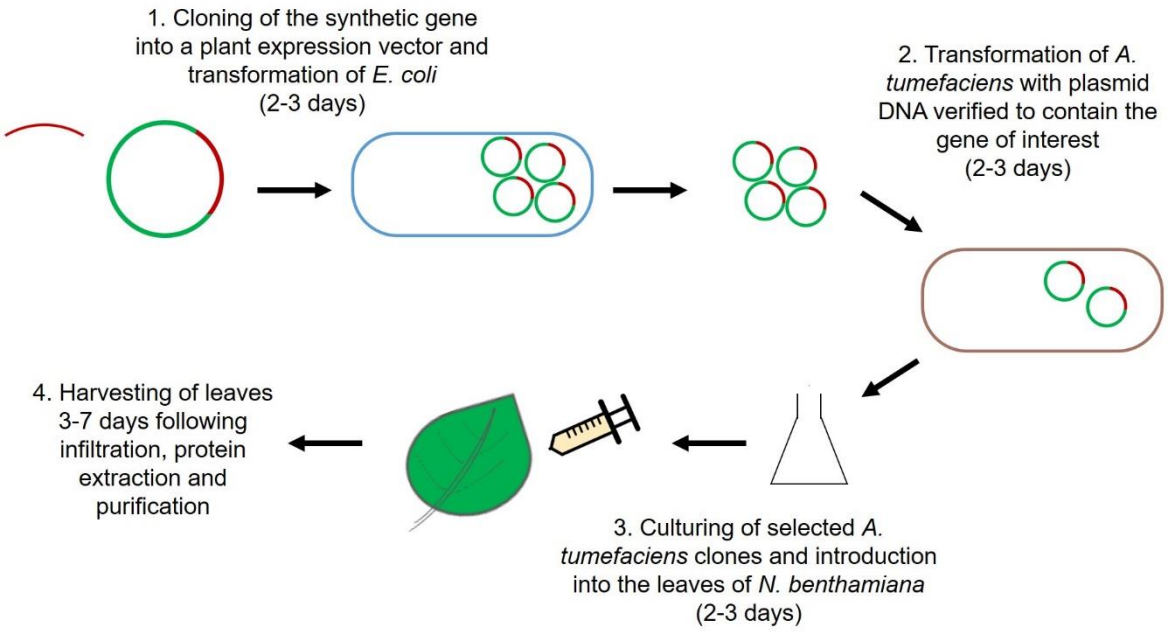


Figure 1.3: A schematic representation of transient plant-based expression via agroinfiltration, using a syringe without a needle. Adapted from http://www.pbltechnology.com/molecular-pharming-high-level-plant-protein-expression-cpmv-ht/.

1.8.2. Factors that influence protein yield during transient plant-based expression

1.8.2.1. Codon optimization and plant expression vectors

Codon optimization is described as the alteration of codons within the gene sequence to enhance recombinant expression (Gustafsson *et al.,* 2004; Webster *et al.,* 2017). Modification of the foreign gene to more closely reflect the codon usage of the host has become widely practised, although the benefits of this process are often inconsistent (Gustafsson *et al.,* 2004; Webster *et al.,* 2017). In a study by Maclean and colleagues (2007), expression of a plant codon optimized version of a gene in *N. benthamiana* was far inferior to expression using a human-codon optimized version and was also less effective in comparison to the native gene. It has been suggested a high GC content may increase translational efficiency through enhanced mRNA stability, processing and nucleocytoplasmic transport, and that the presence of a G or C at the third nucleotide position of a codon is particularly important (de Rocher *et al.,* 1998; Suo *et al.,* 2006; Jiang *et al.,* 2007; Stachyra *et al.,* 2016). Human-codon optimization typically results in a higher GC content, while plant codon optimization typically results in a higher AT content, which could contribute to the differences in expression levels reported. Thus, codon optimization does affect protein expression levels, although optimization for expression in the host species is empirical.

Advanced vector technologies are important contributors to the increasing popularity of transient plant-based expression. The introduction of second-generation plant expression plasmids that are able to carry larger size inserts have overcome issues of low protein yield, biocontainment, and the limitation of transgene size (Gleba *et al.,* 2005; Sainsbury and Lomonossoff, 2008). These "deconstructed vectors" consist only of the foreign gene of interest and the minimum plant virus components required for replication, hence the necessity of *Agrobacterium* to transport the target gene into the plant cell for subsequent transcription and translation. For example, Icon Genetics (Halle (Saale), Germany) has developed a suite of deconstructed pro-vector modules ("magnICON") based on the tobacco mosaic virus (TMV) and potato virus X (PVX) for the rapid expression of high yields of heterologous protein (Marillonnet *et al.,* 2004; Giritch *et al.,* 2006). The combination of these non-competitive vector types facilitates the expression of heteromeric or multiple proteins, which is not facilitated by a monopartite viruses like TMV (Giritch *et al.,* 2006; Sainsbury and Lomonossoff, 2008). This magnifection system entails the co-infiltration with three modules (5', 3', and recombinase) for the *in planta* assembly of a functional viral vector. The use of different 5' modules facilitates targeting of the heterologous protein to different subcellular compartments, which reportedly influences yield (Marillonnet *et al.,* 2004; Maclean *et al.,* 2007; Pêra *et al.,* 2015). Using the magnifection system, protein yields of up to 5 grams per kilogram (kg) fresh weight biomass (accounting for up to 80% of total soluble protein) have been reported for green fluorescence protein in *N. benthamiana,* although such high yields are rarely attained for other proteins (Marillonnet *et al.,* 2004; Gleba *et al.,* 2005; Marillonnet *et al.,* 2005; Giritch *et al.,* 2006; Rybicki, 2014). In contrast to the magnICON vectors, the popular cowpea mosaic virus (CPMV)-based pEAQ-Hyper-Translatable (HT) vector can be used to express multiple proteins within the same cell, which is ideal for plant-based expression of heteromeric (e.g. antibodies) and hetero-multimeric protein complexes like bluetongue VLPs (Sainsbury and Lomonossoff, 2008; Sainsbury *et al.,* 2009; Mokoena *et al.,* 2019) where at least four different capsid proteins need to be expressed and assemble to form a VLP. For expression using the non-replicating pEAQ-HT vector, which contains the tomato bushy stunt virus P19 gene in its backbone to suppress post-transcriptional gene silencing and consequently enhance expression levels, the signal sequence for subcellular localization is incorporated into the foreign gene to be expressed. The pEAQ-HT plant-expression vector permits high-level and rapid transient expression of heterologous proteins in plants, with yields of up to 1.5 g per kg fresh weight biomass (accounting for up to 25% of total soluble protein) reported using *N. benthamiana* as host (Sainsbury and Lomonossoff, 2008; Sainsbury *et al.,* 2009). Thus, the choice of plant expression vector is an important consideration for transient plant-based expression.

1.8.2.2. *A. tumefaciens* strains, culture density and chemical inducers of its virulence genes

Another important factor that can influence the yield and speed of heterologous protein expression is the choice of *Agrobacterium*. A range of disarmed *A. tumefaciens* strains with different selectable marker genes and promoters have been developed and are available for transient plant-based expression (Hellens *et al.,* 2000). These laboratory *A. tumefaciens* strains have variable translational efficiencies and are not equally effective for all proteins and plant hosts (Wroblewski *et al.,* 2005; Wydro *et al.,* 2006; Norkunas, 2014; Shamloul *et al.,* 2014). The addition of chemical additives like acetosyringone, a potent inducer of *Agrobacterium* virulence genes, in the cultivation media and/or infiltration buffer can enhance the transformation efficiency and consequently augment recombinant protein expression (Norkunas, 2014; Pêra *et al.,* 2015). The density of the *A. tumefaciens* culture introduced into the plant leaves is also an important factor to consider. Culture densities that are too low result in low protein expression, while too high densities could result in necrosis of the infiltrated leaves due to a hypersensitive response (Wroblewski *et al.,* 2005; Loh and Wayah, 2014). Thus, the choice of *Agrobacterium* strain and its culture density introduced into the plant host are important considerations in transient expression systems, and the utilization of chemical additives can enhance the transformation efficiency of *Agrobacterium.*

1.8.2.3. Protein extraction, clarification and purification

Downstream processing (DSP), which includes protein extraction, clarification and purification, is very important for plant-based production systems. DSP accounts for a significant portion of the total operating costs for product manufacturing and the removal of contaminants is crucial in ensuring good manufacturing practice (GMP) compliance (Wilken and Nikolov, 2012; Lojewska *et al.,* 2016). In transient expression systems, protein extraction typically starts with harvesting of infiltrated leaves for homogenization by press or blade-based homogenizers (Lojewska *et al.,* 2016). The extraction buffer is critical to obtain the required pH and salinity to improve protein stability, with protease inhibitors usually added to prevent proteolysis of the target protein via host proteases and subsequently enhance recombinant protein yield (Azzoni *et al.,* 2005; Grosse-Holz *et al.,* 2018). A wide variety of biological buffers are available, each with a different pH range where its buffering capacity is at its most effective. Following extraction, the plant extract is clarified via centrifugation and filtration (e.g. two layers of Miracloth™ or cheesecloth) in order to remove soluble contaminants that can interfere with further downstream processes. The purification strategy is tailored for each individual protein based on inherent properties like solubility, size, charge, isoelectric point (pI) and hydrophobicity, and should not contain more than three processing steps to maximize purification efficiency (Chen, 2008; Lojewska *et al.,* 2016). On a

commercial scale, ultrafiltration/diafiltration (e.g. tangential flow filtration (TFF)) and one or more column chromatography steps (e.g. size exclusion, affinity chromatography and ion exchange chromatography) have been reported for the purification of human monoclonal antibodies and VLP vaccines (Landry *et al.,* 2010; Lai and Chen, 2012; O'Hara *et al.,* 2012). For veterinary products, however, the purification requirements are a lot less stringent and the use of ultrafiltration/TFF for concentration and partial purification, as well as the use of clarified plant extract, have been reported for VLP vaccines (Ruiz *et al.,* 2018; Mokoena *et al.,* 2019). Thus, DSP can affect the quality and quantity of the expressed protein, as well as the scalability of manufacturing, and keeping the purification steps to the minimum is vital for the development of a cost-effective product.

1.8.3. The development and production of plant-based proteins and vaccines

The advancement of plant-based products has become a priority and extensive funding has been invested in Biopharming activities (Morris *et al.,* 2017). Two European Union FP7-funded projects, namely the PLAPROVA consortium that focused on plant-based vaccines against diseases such as avian influenza, Bluetongue virus disease and foot-and-mouth disease, and the CoMoFarm that focused on consistent homogenous material and establishing cGMP facilities for plant-based production, has contributed greatly to this cause (Morris *et al.,* 2017). Characterization of the nature of contaminants and proving GMP compliance are some of the major challenges of plant-based production systems (Lojewska *et al.,* 2016). The United States of America Food and Drug Administration (FDA) has approved several plant-based products and many more are in advanced clinical trials, including plant-based VLP vaccines for human health.

One of the global leaders in plant-based VLP vaccines for humans is the biotechnology company Medicago (Quebec City, Canada). Making use of pEAQ-HT, Medicago have produced seasonal and pandemic VLP-based influenza vaccines transiently in *N. benthamiana* (D'Aoust *et al.,* 2008; Landry *et al.,* 2010). Medicago demonstrated that influenza VLPs could be produced from the expression of HA alone, which equates a simple production system that is advantageous in terms of cost and speed of development. Jutras *et al.* (2015) did, however, find that the co-expression of HA and M2 could result in increased protein yield for some subtypes. The plant-based VLP antigens, which were shown to accumulate in the apoplastic indentations of the plasma membrane, showed good safety and immunogenicity in pre-clinical and clinical trials (Phase I or Phase I and II) (Chen *et al.,* 2007; D'Aoust *et al.,* 2008; D'Aoust *et al.,* 2010; Landry *et al.,* 2010). These plant-produced VLPs elicited strong long-lasting antibody responses, as well as potent T-cell mediated immune responses through interaction with antigen presenting cells (particularly dendritic cells)

(Bright *et al*., 2007; Quan *et al*., 2007; Song *et al.*, 2010; Landry *et al.,* 2014; Shoji *et al*., 2015). In addition, these plant-produced HA VLPs showed potential for cross-protection against other HA subtypes (Landry *et al.,* 2010; Landry *et al.,* 2014). Once the HA sequence of the latest influenza strain was available, 30 million doses of the VLP vaccine could be produced within 3 months (D'Aoust *et al.,* 2008; D'Aoust *et al.,* 2010; Margolin *et al*., 2018). Thus, HA-based VLPs produced in plants are efficacious and advantageous in terms of speed of production, cost and safety. The use of plant-based influenza VLP vaccines have not been tested for the poultry industry.

1.9. Aims and Objectives of this study

Given the numerous advantages of plant-produced VLPs as vaccines, it is reasonable to consider such a platform for the production of a new cost-effective vaccine against H6 AI for poultry in South Africa. Due to the short production time, this platform would facilitate the periodic updating of AI vaccines as recommended by the OIE, to ensure effective protection of vaccinated birds against circulating field strains. In this study, as a proof of concept, sub-lineage I and II HA-based H6 VLPs were transiently produced in *N. benthamiana* plants and tested for efficacy in SPF chickens against a H6N2 field isolate, in comparison to the commercial inactivated H6N2 vaccine. The aim of this study was, therefore, to develop and produce a plant-based VLP vaccine against sub-lineage I and II H6 avian influenza, respectively, for chickens in South Africa.

This study consisted of two primary objectives, the first of which being the development and production of sub-lineage I H6 influenza VLPs transiently in *N. benthamiana* plants using the plant expression vector pEAQ-HT. To maximize protein yield, the appropriate *A. tumefaciens* strain*,* culture density (OD_{600}) of the infiltration suspension and extraction buffer were selected and the effect of codon optimization and co-expression with influenza M2 on expression levels were determined. A combination of several molecular and serology techniques were employed to characterize the plant-based H6 VLPs and to determine the optimal production conditions, including sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, negative-staining transmission electron microscopy (TEM), liquid chromatography-mass spectrometry (LC-MS/MS)-based peptide sequencing, hemagglutination and hemagglutination inhibition (HI) assays. The selected conditions were subsequently utilized for the production of sub-lineage II H6 influenza VLPs in *N. benthamiana.* In addition, in order to investigate the feasibility of protein purification via immobilized metal affinity chromatography (IMAC), a poly-histidine (His)-tag was added to the H6 sub-lineage I and II HA genes. The effect of the addition of the His-tag on protein

expression levels and VLP assembly were investigated using the above-mentioned techniques.

The second objective was to determine the efficacy of the plant-produced H6 sub-lineage I and II VLPs in prime-boost vaccinated SPF White Leghorn chickens against challenge with a heterologous 2016 H6N2 field isolate (sub-lineage I). The primary determinant of vaccine efficacy was the capacity to effectively reduce shedding following viral challenge. Viral shedding titers from the respiratory and gastrointestinal tracts were determined at specific time points from two to twenty-one days post challenge using a qRT-PCR assay targeting conserved regions of the influenza matrix gene (Spackman *et al.,* 2003). In addition, humoral immune responses (HA- and NP-specific antibody responses) were evaluated after the primary immunization, after the secondary immunization, and two weeks after viral challenge using HI assays and commercially available IDEXX Influenza A Ab test kits. For both the sub-lineage I and II H6 VLP vaccines the qRT-PCR and serology results were compared to that of the commercial inactivated vaccine group and the non-vaccinated control group, respectively.

Chapter 2

Design, expression and optimization of influenza H6 VLP production in *N. benthamiana*: Pre-infiltration

2.1. Introduction

Plant molecular farming for the production of high-value recombinant proteins has become one of the major applications in applied biotechnology in recent years (Park and Wi, 2016; Rybicki, 2014; Morris *et al.,* 2017). Transient plant-based production systems, which makes use of RNA plant virus-based vectors and *Agrobacterium*-mediated delivery of the foreign gene to be expressed, frequently in *Nicotiana benthamiana*, are usually preferred over stable genetic transformation due to a reduction in development time and production time (Goodin *et al.,* 2008; Sainsbury and Lomonossoff, 2008). Transient plant-based production systems are highly scalable and cost-effective, has a low risk of transmitting a pathogen to humans, facilitates glycosylation of the target protein and has a short production time, with recombinant protein accumulation usually peaking within 3 to 7 days post infiltration (Schöb *et al.,* 1997; Goodin *et al.,* 2008; Shamloul *et al.,* 2014). The short production time of plant-based production systems facilitates quick response to outbreaks of emerging diseases (e.g. influenza pandemics) or in the case of bioterror threats (D'Aoust *et al.,* 2010; Morris *et al.,* 2017). Transient plant-based expression has been widely adopted for the production of pharmaceutically important and commercially valuable proteins like antibodies, enzymes, hormones and vaccine antigens, including influenza virus-like particle (VLP) vaccines (Chen, 2008; D'Aoust *et al.,* 2008; Landry *et al.,* 2010; Shoji *et al.,* 2011; Rybicki, 2014; Shoji *et al.,* 2015; Sack *et al.,* 2015).

Influenza vaccines have been one of the major success stories of transient plant-based expression. Vaccination against zoonotic diseases like influenza is in support of the "One Health Initiative", which is a worldwide strategy that integrates human medicine, veterinary medicine and environmental science to enhance the health and well-being of all species (http://www.onehealthinitiative.com/mission.php). Seasonal and pandemic VLP-based influenza vaccines for humans have been produced in *N. benthamiana* from the singular expression of hemagglutinin (HA), although the co-expression of HA and influenza matrix 2 ion channel (M2) could result in increased protein yield for some subtypes (D'Aoust *et al.,* 2008; Jutras *et al.,* 2015). These plant-based influenza VLPs were produced rapidly at low cost, showed good safety and immunogenicity (humoral and cellular responses) in pre-clinical and clinical tests (Phase I and II), and showed potential for cross-protection against other HA subtypes (D'Aoust *et al.,* 2008; D'Aoust *et al.,* 2010; Landry *et al.,* 2010; Landry *et*

al., 2014). The short production time frame of transient plant-based expression systems is especially important in the case of viruses with a high mutation rate (e.g. influenza A) that require vaccines to be updated frequently or in the face of a pandemic (D'Aoust *et al.,* 2010). The use of plant-produced influenza VLP vaccines is untested for the poultry industry.

Low pathogenic avian influenza (LPAI) H6N2 is one of the most prevalent subtypes in poultry and is endemic in many regions, including South Africa (Chin *et al.,* 2002; Liu *et al.,* 2003; Woolcock *et al.,* 2003; Abolnik, 2007a; Abolnik, 2007b; Alexander, 2007; Brown, 2010; Wang *et al.,* 2014; Wang *et al.,* 2016). In South Africa the infection started in chickens in the early 2000s and two related but distinct viral sub-lineages were identified from the onset (Abolnik, 2007a). A registered egg-based inactivated vaccine (AVIVAC® AI; Deltamune (Pty) Ltd., South Africa) derived from a H6N2 sub-lineage I virus isolated in 2002 has been used to control sporadic outbreaks in commercial flocks and is still in use at the time of writing. Extensive evolutionary changes have been documented for H6N2 field viruses after more than a decade of vaccination, especially in sub-lineage I isolates, and antigenic mismatching between the vaccine and field strain has been shown to reduce the vaccine's capacity to protect against viral shedding (Rauff *et al.,* 2016; Abolnik *et al.,* 2019; Swayne and Kapczynski, 2017). The effectiveness of the commercial vaccine against recent circulating strains have not been demonstrated in a clinical study, nor have any subsequent field isolates been developed as replacement vaccine seed strains.

In this chapter, transient plant-based expression was utilized for the production of VLPs against H6 avian influenza for chickens in South Africa. H6 sub-lineage I and II VLPs, based on the full-length hemagglutinin (HA_0) sequences of H6N2 viruses isolated from chickens in South Africa in 2016 and 2012, respectively, were transiently produced in *N. benthamiana* modified to prevent plant-specific glycosylation of the target protein, using the non-replicating cowpea mosaic virus (CPMV)-based pEAQ-HT plant expression vector (Sainsbury and Lomonossoff, 2008; Strasser *et al.,* 2008). As the commercial viability of a transiently expressed plant-based recombinant protein is dependent on the yield obtained, several factors that reportedly influence expression levels were investigated to maximize protein yield (Wydro *et al.,* 2006; Maclean *et al.,* 2007; Norkunas, 2014; Pêra *et al.,* 2015). This chapter focuses on the design, expression and optimization of the transient production of H6 VLPs in *N. benthamiana*, up to the point of infiltration, including selecting the optimal codon optimization strategy, *Agrobacterium tumefaciens* strain and culture density, as well as determining the effect of co-expression with the influenza ion channel protein M2 and the addition of the chemical additive acetosyringone to elevate expression levels. Used in

combination, these optimized conditions will lead to enhanced expression levels of sub-lineage I H6 VLPs in *N. benthamiana* and consequently contribute to the cost-effectiveness of this product. The sub-lineage I H6 HA_0 construct was used for the initial optimization, after which the optimized conditions were selected for the expression of sub-lineage II H6 VLPs.

2.2. Materials and methods

2.2.1. Ethical considerations

The general project was approved by the Faculty of Veterinary Science Research Ethics Committee, University of Pretoria (UP). Prior to commencement of laboratory work, approval was obtained from the Council for Scientific and Industrial Research (CSIR) Research Ethics Committee for the cloning of capsid proteins into plant expression vectors and for the production of the H6 VLP AI candidate vaccine in plants (Veterinary Biologicals (Ref: 90/2014) (Appendix A)).

2.2.2. Construction and validation of expression vectors

2.2.2.1. Gene design and synthesis

A synthetic gene was designed based on the full-length hemagglutinin gene sequence of a LPAI H6N2 AI virus (A/chicken/South Africa/N2826/2016 (sub-lineage I); GenBank: MH170289) (Appendix B). The native signal peptide sequence was replaced by a sequence encoding the *Mus musculus* monoclonal antibody heavy chain variable region signal peptide (O'Hara *et al.,* 2012). The replacement of the native signal peptide sequence is common practise in recombinant protein expression, including the expression of human influenza VLPs in plants (D'Aoust *et al.,* 2008). As the murine signal peptide sequence was found to work well for previous projects at the CSIR involving plant-based expression of different types of recombinant protein, it was, therefore, selected for use in this study as well. Due to funding constraints, the use of the native signal peptide sequence was not included as comparison. In addition, to allow cloning into the non-replicating cowpea mosaic virus (CPMV)-based pEAQ-HT plant expression vector, *Age*I and *Xho*I restriction enzyme recognition sites were incorporated at the 5'- and 3'-terminals, respectively. The modified gene was codon optimized by Bio Basic Canada Inc. for expression in chickens (*Gallus gallus*), humans (Human NEW II) and plants (*N. benthamiana*), respectively, in order to evaluate translational efficiency of the resulting protein. In this chapter, $H6_{HA}$S1C, $H6_{HA}$S1H and $H6_{HA}$S1P refers to the synthetic chicken, human and plant codon optimized genes, respectively (Figure 2.1).

In order to investigate the effect of co-expression with the influenza matrix protein 2 (M2) ion channel protein, a synthetic gene based on the M2 gene segment of an H1N1 influenza A virus (A/New Caledonia/20/1999 (H1N1); Gen Bank: HQ008884.1) was used (Appendix B; Jutras *et al.,* 2015). This synthetic M2 gene was previously designed by colleagues to contain the above-mentioned murine signal peptide sequence, as well as *Age*I and *Xho*I restriction enzyme recognition site at the 3'- and 5'-terminals, respectively (Figure 2.1). The modified M2 gene was codon optimized by Bio Basic Canada Inc. for expression in plants (*N. benthamiana*).

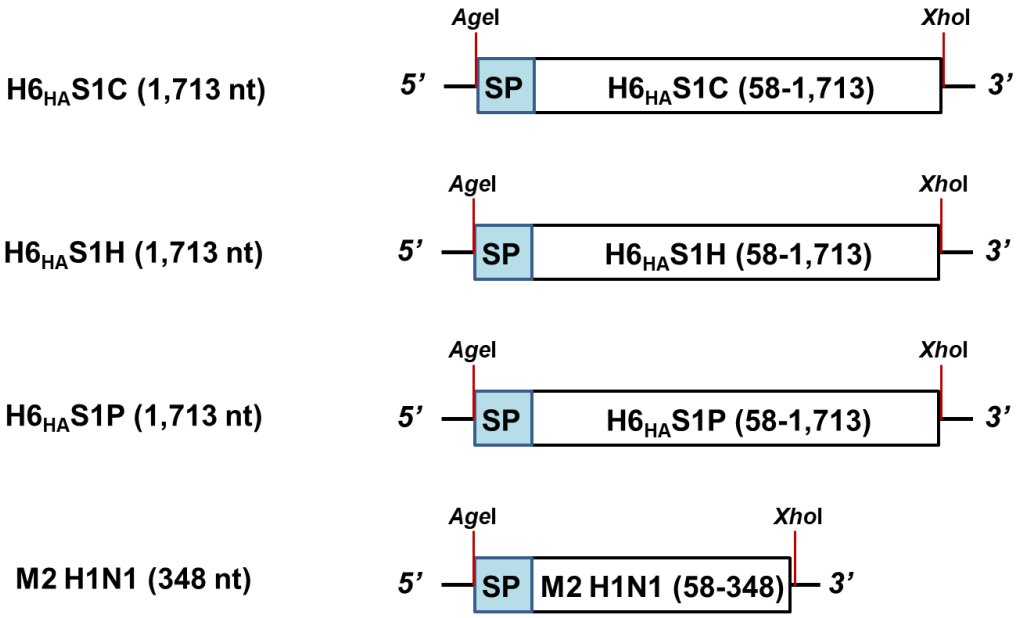


Figure 2.1: Schematic representation of the gene inserts used in this chapter. The full-length sub-lineage I hemagglutinin (HA_0) gene was codon optimized for expression in chickens ($H6_{HA}S1C$), humans ($H6_{HA}S1H$) and plants ($H6_{HA}S1P$), respectively, while the M2 (H1N1) gene was codon optimized for expression in plants. The murine signal peptide (SP) sequence was incorporated at the N-terminus, and *Age*I and *Xho*I restriction enzyme recognition sites were incorporated at the N- and C-termini for cloning into the plant expression vector pEAQ-HT. For each gene, the nucleotide (nt) numbering (as determined from the start codon to the termination codon) is provided in brackets.

2.2.2.2. Cloning of the H6 HA_0 gene fragments into the pEAQ-HT plant expression vector

For the cloning of $H6_{HA}S1C$, $H6_{HA}S1H$, and $H6_{HA}S1P$ genes into pEAQ-HT, plasmid DNA obtained from Bio Basic Canada Inc. were digested with *Age*I and *Xho*I (Thermo Fisher Scientific™, Waltham, Massachusetts, United States of America) restriction enzymes to excise the respective synthetic genes from the carrier vector (pUC57). Each of the synthetic genes were subsequently inserted into pEAQ-HT digested with *Age*I and *Xho*I, using the Fast-Link DNA Ligase kit (Epicentre, Madison, Wisconsin, United States of America) according to the manufacturers' instructions. A summary of the plasmids constructed and/or used in this chapter is provided in Table 2.1.

Table 2.1: The expression vectors constructed and used in this chapter.

Constructed vector	Insert description
pEAQ-HT+$H6_{HA}S1C$	Chicken codon optimized synthetic H6 HA_0 gene (A/chicken/South Africa/N2826/2016 (H6N2))
pEAQ-HT+$H6_{HA}S1H$	Human codon optimized synthetic H6 HA_0 gene (A/chicken/South Africa/N2826/2016 (H6N2))
pEAQ-HT+$H6_{HA}S1P$	Plant codon optimized synthetic H6 HA_0 gene (A/chicken/South Africa/N2826/2016 (H6N2))
pEAQ-HT+M2	Plant codon optimized synthetic H1N1 M2 gene (A/New Caledonia/20/1999 (H1N1))

HA: hemagglutinin; M2: matrix protein 2

2.2.2.3. Transformation of competent DH10B with constructed plasmids

Making use of electroporation (Gene-Pulser™ Bio-Rad, Hercules, California, United States of America; 1.8 kV, 25 µF, 200 Ω), the ligation reaction mixtures described in section 2.2.2 and the pEAQ-HT expression vector without an insert (negative control) were introduced into competent *Escherichia coli (E. coli)* cells (DH10B). The bacterial cells were subsequently re-suspended in 800 µl super optimal broth with catabolite repression (SOC) (2% [w/v] tryptone (Oxoid™, supplied by Thermo Fisher Scientific™), 0.5% [w/v] yeast extract (New England BioLabs, supplied by Inqaba Biotec (Pty) Ltd., Pretoria, South Africa), 10 mM sodium chloride (NaCl) (Associated Chemical Enterprises (Pty) Ltd., Johannesburg, South Africa), 2.5 mM potassium chloride (KCl), 10 mM magnesium chloride ($MgCl_2$), 20 mM magnesium sulfate ($MgSO_4$), and 20 mM glucose; pH 7.0) and incubated for 1 hour at 37°C, with agitation (200 rpm). For selection, the transformed cells were plated onto Lysogeny

broth (LB) agar (1% [w/v] NaCl, 0.5% [w/v] yeast extract, 1% [w/v] tryptone, and 1.5% [w/v] bacteriological agar; pH 7.0) supplemented with 50 µg/ml kanamycin and incubated overnight at 37°C. Clones resistant to antibiotics were selected for verification using colony PCR and Sanger DNA sequencing. Chemicals were from Sigma-Aldrich (St. Louis, Missouri, United States of America) now MERCK (Pty) Ltd. (Darmstadt, Germany), unless otherwise stated.

For colony PCR, the PCR reactions were performed in a Mastercycler® EP gradient S (Eppendorf, Hamburg, Germany). The KAPA2G™ Robust PCR kit (KAPA Biosystems, Cape Town, South Africa) and pEAQ-HT-specific primers (forward primer: 5'-ACTTGTTACGATTCTGCTGACTTTCGGCGG-3'; reverse primer: 5'-CGACCTGCTAAACAGGAGCTCACAAAGA-3') were utilized. The PCR reactions consisted of the following: 4 µl of 5 X KAPA 2G buffer, 0.4 µl dNTP mix, 0.2 µM forward and reverse primer, respectively, 0.1 µl of KAPA2G Robust DNA polymerase (5 U/µl), bacterial culture as template, and sterile nuclease-free water to a final volume of 20 µl. The cycling conditions were as follows: 1 cycle at 95°C for 2 minutes, 35 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 45 seconds, with a final extension step of 72°C for 5 minutes. PCR products were resolved on a 1% [w/v] agarose gel in 1 x TAE buffer (40 mM Tris (Tris(hydroxymethyl)aminomethane), 20 mM acetic acid, 1 mM EDTA, pH 8.0) at 120 V for 30 minutes. For selected positive clones, the respective PCR products were excised from the agarose gel and purified using the Zymoclean gel DNA recovery kit (Zymo Research, Irvine, California, United States of America) according to manufacturer's instructions. Purified PCR product was subsequently verified via Sanger DNA sequencing (Inqaba Biotec™, Pretoria, South Africa).

2.2.3. Transformation of the verified constructed expression vectors into *Agrobacterium tumefaciens* and validation of the transformants

Three commonly used *A. tumefaciens* strains, namely AGL-1, GV3101::pMP90 and LBA4404 (Hellens *et al.,* 2000), were compared in this study. *Agrobacterium* strain AGL-1 was imported with a permit (P0079617) issued by the South African Department of Agriculture, Forestry and Fisheries (DAFF) and from the American type culture collection (ATCC® BAA-101™, *Rhizobium radiobacter*), GV3101::pMP90 was a kind gift from Dr Csaba Koncz, Max-Planck Institut für Züchtungsforschung, Germany, and Invitrogen™ ElectroMAX™ LBA4404 was purchased (Thermo Fisher Scientific™). Following selection of positive recombinant clones in section 2.2.2.3, electroporation (1.44 kV, 25 µF, 200 Ω) was employed to transform each of the verified plasmids into competent *A. tumefaciens* cells.

The bacterial cells were subsequently re-suspended in 800 µl of LB (1% [w/v] NaCl, 0.5% [w/v] yeast extract, and 1% [w/v] tryptone, pH 7.0) and incubated for 3 hours at 28°C with agitation (200 rpm). For selection, the transformed cells were spread-plated onto LB agar supplemented with appropriate selective antibiotics (30 µg/ml rifampicin, 50 µg/ml kanamycin and either 50 µg/ml carbenicillin (AGL-1), 10 µg/ml gentamycin (GV3101::pMP90), or 50 µg/ml streptomycin (LBA4404)) and incubated over two nights at 28°C. Clones resistant to antibiotics were selected for verification via colony PCR (as described in section 2.2.2.3). Thereafter, for further validation, plasmid DNA isolated from selected *Agrobacterium* clones were transformed back into competent DH10B *E. coli* cells for verification via colony PCR and Sanger DNA sequencing as before.

2.2.4. Infiltration of *N. benthamiana* with *A. tumefaciens* transformants

Agroinfiltration was used to infiltrate plant leaves with validated transformed *A. tumefaciens* strains GV3101::pMP90, AGL-1 and LBA4404 harbouring the constructed vectors (Table 2.1) (Shamloul *et al.,* 2014). Validated *A. tumefaciens* cultures were streaked onto fresh LB containing appropriate selective antibiotics. The appropriate growth medium containing selective antibiotics (30 µg/ml rifampicin and 50 µg/ml kanamycin) were inoculated with freshly grown plate cultures and incubated overnight at 28°C with agitation (200 rpm) on a rotary shaker. AGL-1 and GV3101::pMP90 clones were grown in LB media, while LBA4404 clones were grown in yeast mannitol broth (YMB) (0.01% [w/v] NaCl, 0.1% [w/v] yeast extract, 1% [w/v] mannitol, 0.0096% [w/v] $MgSO_4$, and 0.038% [w/v] dipotassium phosphate (K_2HPO_4); pH 7.0). Overnight grown Agrobacteria were centrifuged (8,000 x g, 8 minutes) (Avanti J-26 XPI, Beckman Coulter, Brea, California, United States of America) and the respective pellets suspended in infiltration buffer (10 mM 2-N-morpholino-ethanesulfonic acid (MES), 20 mM $MgSO_4$, pH 5.6) supplemented with 200 µM acetosyringone, unless indicated otherwise. The respective infiltration mixes were diluted to obtain a final optical density at 600 nm (OD_{600}) of 1.5, unless indicated otherwise. Following incubation at room temperature for at least one hour, the *A. tumefaciens* suspensions were introduced into mature leaves of glyco-engineered *N. benthamiana* plants (Strasser *et al.,* 2008) using a syringe without a needle. The plants were returned to the growth rooms until harvest.

2.2.5. Harvesting of infiltrated leaf material and total protein extraction

At 6 days post infiltration (dpi) (unless otherwise indicated) the total soluble protein (TSP) was extracted from the plant leaf tissue by homogenization and the target protein purified. Plant material was crushed to a fine pulp using a Matstone DO9001 Juicer in two volumes of cold Tris extraction buffer (50 mM Tris, 150 mM NaCl, and 0.04% sodium metabisulfite, pH

8.0) (Landry *et al.,* 2010). Prior to use, the extraction buffer was supplemented with proteinase inhibitor cocktail (P2714, Sigma-Aldrich) to prevent proteolysis of the target protein via host proteases (Azzoni *et al.,* 2005; Grosse-Holz *et al.,* 2018). After homogenization, the slurries were clarified through a double layer of cheese cloth and the plant extract centrifuged (7,000 x g, 7 minutes, 4°C). Target protein was subsequently purified using differential centrifugation purification, which is suitable for small laboratory-scale experiments. The clear supernatant containing H6-VLPs was loaded on top of a freshly prepared 20 to 60% Iodixanol (OptiPrep™ density gradient medium) density gradient (Figure 2.2). Layers 30% and 40% were 1.5 ml each, while all other layers were 1 ml each. Following ultracentrifugation (32,000 x g, 2 hours, 10°C; Beckman Coulter Ultra-centrifuge Optima L90K), fractions of 500 µl each were collected from the bottom of the Thinwall Ultra-Clear™ tube (Beckman Coulter).

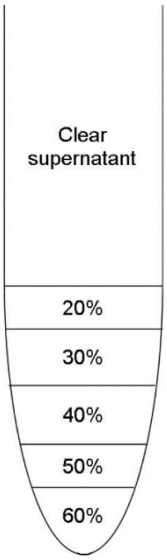


Figure 2.2: A schematic representation of an Iodixanol (OptiPrep™) density gradient ranging from 20% to 60%, overlaid with the clear supernatant of the plant extract.

2.2.6. Biochemical analysis and confirmation of identity

2.2.6.1. Gel electrophoresis and immunoblotting

The partially-purified protein was analyzed using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. For SDS-PAGE analysis, protein was separated on an Invitrogen Bolt™ 4-12% Bis-Tris Plus gel (Thermo Fisher Scientific™) under reducing conditions and stained with Coomassie G-250. For confirmation of identity, clearly visible SDS-PAGE bands corresponding to the expected fragment size of each of the target proteins were excised and analyzed by LC MS/MS-based peptide sequencing. For

immunoblotting, protein was separated on a 10% TGX Stain-Free™ FastCast™ acrylamide gel (Bio-Rad) under reducing conditions and transferred to an Immubilon PVDF membrane using the semi-dry transblot Turbo blotter (Bio-Rad), according to manufacturer's recommendation. Blocking was performed in 1 x phosphate buffered saline (PBS) (4.3 mM sodium phosphate ($NaHPO_4$), 1.4 mM monopotassium phosphate (KH_2PO_4), 2.7 mM KCl, 127 mM NaCl; pH 7.4) containing Tween 20 (0.1% [v/v]) and 3% [w/v] Bovine Serum Albumin Fraction V (BSA) for at least two hours. H6 HA_0 purified using differential centrifugation was detected using AI H6N2 antiserum (1:600; Deltamune Pty (Ltd), Pretoria, South Africa) and goat anti-chicken IgY horseradish peroxidase (HRP) conjugated antibody (1:1,500; Novex Life Technologies, A16054; Thermo Fisher Scientific™). Chemiluminescence detection (Clarity™ Western ECL Blotting Substrate; Bio-Rad) and the ChemiDoc™ MP Imaging System (Bio-Rad) were used for protein visualization, as recommended by the manufacturer.

2.2.6.2. Transmission electron microscopy (TEM)

To confirm the formation of HA-based VLPs, partially-purified H6 VLPs were prepared for examination by negative-staining transmission electron microscopy (TEM) (Electron Microscopy Unit, UP). Carbon-coated copper grids (mesh size 200) were floated on 15 µl of the density gradient-purified fractions for 5 minutes and washed by floating on 5 µl sterile water for a total of five washes. Particles were subsequently negatively-stained with 2% uranyl acetate for 30 seconds. A Philips CM10, 80 kV transmission electron microscope was used for imaging.

2.2.6.3. Hemagglutination and hemagglutination inhibition (HI) tests

To confirm the presence of functional HA-based VLPs, plant-produced H6 VLPs were tested using hemagglutination and hemagglutination inhibition assays. The assays were performed at the South African National Accreditation System (SANAS) accredited Department of Veterinary Tropical Diseases, Serology Laboratory, UP, according to guidelines (The World Organisation for Animal Health (OIE), 2018). For the hemagglutination assay, two-fold serial dilutions of the plant-produced VLPs were tested for their ability to agglutinate chicken erythrocytes. The HA titer was calculated as the reciprocal of the dilution that shows complete agglutination of the chicken erythrocytes (referred to as one hemagglutinating unit (1 HAU)). HA titers were considered to be positive if complete agglutination of chicken erythrocytes was observed at a sample dilution of 1/16 (2^4 or 4 $\log_2$ when expressed as the reciprocal) or more. Based on the HA titer, four hemagglutinating units (4 HAUs) of each positive sample was subjected to HI analysis. For HI, two-fold serial dilutions of each prepared sample (calculated to contain 4 HAUs of antigen), were tested against H6N2 AI

positive antisera, or AI negative antiserum (negative control). The end-point of the titration was the highest dilution wherein hemagglutination was completely inhibited. HI titers were considered to be positive if complete inhibition of hemagglutination was observed at a sample dilution of 1/16 (2^4 or 4 $\log_2$ when expressed as the reciprocal) or more. However, as low non-specific reactions were observed with the HI assay against the negative control SPF sera, likely due to the presence of other plant proteins, the absolute HI titer is consequently difficult to determine.

2.2.6.4. Liquid chromatography-mass spectrometry (LC-MS/MS)-based peptide sequencing

Liquid chromatography-mass spectrometry (LC-MS/MS)-based peptide sequencing was performed at CSIR Biosciences, Pretoria, to confirm the presence of the target protein(s). Clearly visible SDS-PAGE protein bands corresponding to the expected size of the HA_0 fragment (± 62 kDa) and/or M2 (± 11 kDa) were excised, in-gel trypsin digested (Shevchenko *et al.,* 2007) and then analyzed as reported by Chhiba-Govindjee *et al.* (2018).

2.3. Results and discussion

2.3.1. Plant-produced H6 VLPs and the effect of codon optimization, *Agrobacterium* strains and co-expression with influenza M2 on protein yield

In this study, functional H6 VLPs that resemble native influenza viral particles were transiently produced in glyco-engineered *N. benthamiana* plants (Strasser *et al.,* 2008). LC-MS/MS-based peptide sequencing results confirming the presence of the H6 HA_0 and M2 ion channel proteins are provided in Appendix C. The H6 VLPs were pleiomorphic but roughly spherical (Figure 2.3) and a wide range in particle size was observed using transmission electron microscopy, which is similar to previous reports regarding plant-based influenza VLPs (D'Aoust *et al.,* 2008; Lindsay *et al.,* 2018). Most of the H6 VLPs measured between 70 and 100 nm in diameter, although particles ranging from 40 to 190 nm were observed. These H6 VLPs successfully agglutinated chicken erythrocytes and the HA titer obtained correlated with the target protein yield detected via immunodetection (Table 2.2; Figure 2.4). Functionality was further confirmed by testing the capacity of these H6 VLPs (diluted to contain 4 HAUs of antigen) to inhibit agglutination in the presence of H6N2 specific antibodies, which yielded high HI titers ranging from 256 (2^8 or 8 $\log_2$) to 1024 (2^{10} or 10 $\log_2$) (Table 2.2).

In order to determine the effect of codon optimization on transgene expression, a full-length synthetic H6 hemagglutinin gene sequence was codon optimized for expression in chickens ($H6_{HA}$S1C), humans ($H6_{HA}$S1H) and plants ($H6_{HA}$S1P), respectively. Of these, only the chicken and human codon variants generated detectable levels of the target protein using SDS-PAGE (Figure 2.4), with the accumulation of target protein peaking at 6 days post infiltration (dpi). Similarly, using agroinfiltration, Maclean *et al.,* (2007) reported that the highest accumulation of the target protein (human papillomaviruses capsid) (17% of TSP) was obtained with a human codon optimized gene variant, while plant codon optimization failed to yield detectable protein (<1% TSP). It has been suggested that a high overall G+C content could increase translational efficiency through enhanced mRNA stability, processing and transport, and that the presence of a G or C at the third nucleotide position of a codon is of particular importance (de Rocher *et al.,* 1998; Suo *et al.,* 2006; Jiang *et al.,* 2007; Stachyra *et al.,* 2016). In this study, the chicken, human and plant codon optimized genes had an overall GC content of 51.52%, 56.32%, 36.86%, respectively, and 67.89%, 80.70% and 23.86% of codons, respectively, had a G or C at the third nucleotide position. Thus, it is possible that lower G+C content could have contributed to the low yield of the target protein obtained with plant codon optimization in comparison to the other optimization strategies.

The bacterial virulence and transformation efficiency of three laboratory strains of *Agrobacterium* available for use at the CSIR, namely GV3101::pMP90, AGL-1 and LBA4404, were compared. Concurrently, in order to determine if the co-expression with the influenza M2 ion channel protein could result in increased protein yield for the H6 subtype (Jutras *et al.,* 2015), each of the vector clones were expressed without and with synthetic plant codon optimized M2 from strain A/New Caledonia/20/1999 (H1N1). For the chicken codon optimized variant, H6 HA_0 were successfully produced using all three *A. tumefaciens* strains, with AGL-1 resulting in the highest yield (Table 2.2; Figure 2.4). Furthermore, co-expression with M2 resulted in increased H6 HA_0 expression when mediated by *A. tumefaciens* strains GV3101::pMP90 and AGL-1. For the human codon optimized gene, H6 HA_0 were detected when mediated by *A. tumefaciens* strains GV3101::pMP90 and LBA4404 only (Table 2.2; Figure 2.4), despite several attempts using different validated positive AGL-1 clones. With human codon optimization, the highest accumulation of target protein was obtained using *A. tumefaciens* strain LBA4404, with or without co-expression with M2 (Table 2.2; Figure 2.4). Thus, in this study, the codon optimization strategy, *Agrobacterium* strain and co-expression with M2 significantly effects transgene expression. Based on these results, as well as taking into account the difficulty of infiltrating LBA4404 transformants at the selected culture density

(OD_{600} = 1 to 2), the chicken codon optimization strategy, *A. tumefaciens* strain AGL-1 and co-expression with M2 was selected for the production of H6 VLPs in plants.

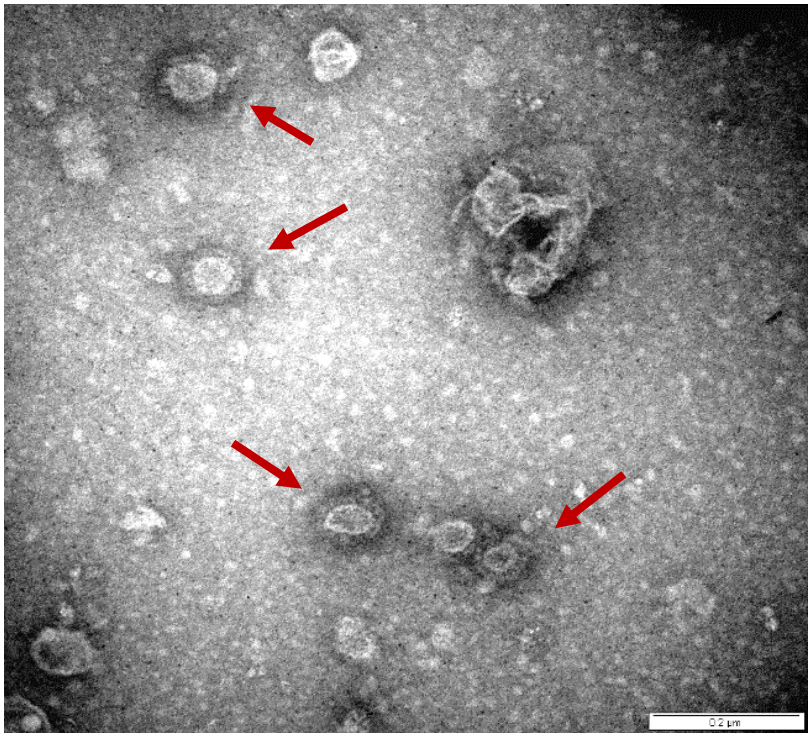


Figure 2.3: Negatively-stained transmission electron microscopy image of sub-lineage I H6 avian influenza VLPs. The chicken codon optimized H6 HA_0 was co-expressed with the M2 ion channel protein, mediated by *A. tumefaciens* strain AGL-1. The arrows indicate the influenza VLPs. The bar represents 0.2 µm.

Table 2.2: Level of protein expression obtained for each of the codon optimization strategies using different *Agrobacterium* strains, when expressed on its own or co-expressed with the influenza M2 ion channel protein.

Codon optimized gene	***Agrobacterium* strain**	**Co-expressed with influenza M2**	**Method of analysis**			
			IB*	**TEM***	**HA titer (4 HAU)**	**HI titer (log_2)**
Chicken	GV3101::pMP90	No	+	+	8	10
		Yes	++	++	256	9
	AGL-1	No	++	+++	64	10
		Yes	+++	+++	512	9
	LBA4404	No	+++	++	256	9
		Yes	++	++	256	8
Human	GV3101::pMP90	No	++	+	16	9
		Yes	+++	++	512	9
	AGL-1	No	-	-		
		Yes	-	-		
	LBA4404	No	+++	++	512	10
		Yes	+++	++	512	10
Plant	GV3101::pMP90	No	-	-		
		Yes	-	-		
	AGL-1	No	-	-		
		Yes	-	-		
	LBA4404	No	-	-		
		Yes	-	-		

- not detected; + detected; ++ detected with confidence; +++ detected in abundance. IB - Immunoblot, TEM – transmission electron microscopy, HA – hemagglutination assay, HAU – hemagglutinating units; HI – hemagglutination inhibition assay.

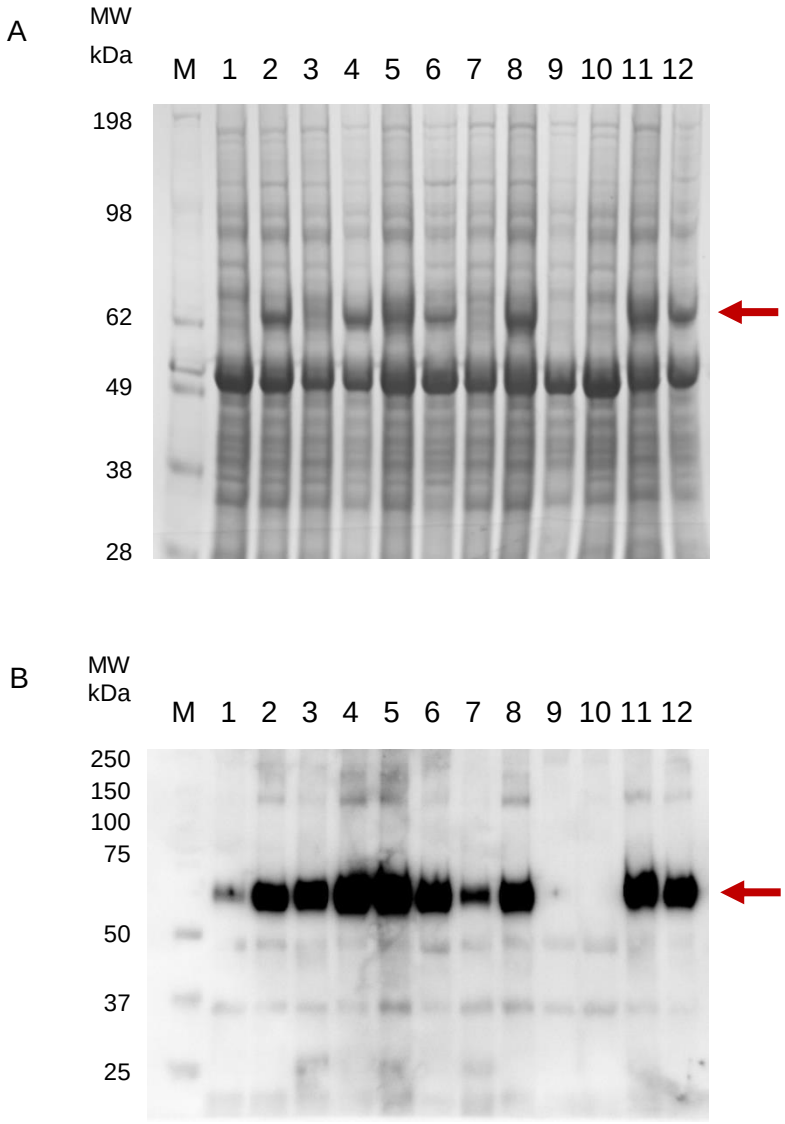


Figure 2.4. SDS-PAGE (A) and immunoblot (B) images demonstrating the impact of codon optimization, *Agrobacterium* strain, and co-expression with the M2 ion channel protein on the yield of sub-lineage I H6 HA_0. Lanes 1 & 2: GV3101::pMP90 mediated expression of $H6_{HA}$S1C, without and with M2, respectively; lanes 3 & 4: AGL-1 mediated expression of $H6_{HA}$S1C without and with M2, respectively; lanes 5 & 6: LBA4404 mediated expression of $H6_{HA}$S1C without and with M2, respectively; lanes 7 & 8: GV3101::pMP90 mediated expression of $H6_{HA}$S1H without and with M2, respectively; lanes 9 & 10; AGL-1 mediated expression of $H6_{HA}$S1H without and with M2, respectively; lanes 11 & 12: LBA4404 mediated expression of $H6_{HA}$S1H without and with M2, respectively. M: The SeeBlue Plus 2 (A) and WesternC (B) protein molecular weight markers were used for the SDS-PAGE and immunoblot, respectively. The arrows indicate the position of the target protein (approximately 62 kDa).

2.3.2. The effect of acetosyringone on protein yield

Acetosyringone is a potent inducer of *Agrobacterium* virulence genes and the addition thereof in the cultivation medium and/or infiltration buffer reportedly plays an important role in plant-based expression (Hiei *et al.,* 1994; Jeoung *et al.,* 2002; Wydro *et al.,* 2006; Norkunas, 2014). In general, the level of protein expression correlated with increasing concentrations of acetosyringone, up to a concentration of 450 - 500 µM (Wydro *et al.,* 2006; Norkunas, 2014). Making use of the pEAQ-HT expression vector, Norkunas (2014) reported a significant increase in the expression of beta-glucuronidase (GUS) with increasing acetosyringone concentrations (200 to 500 µM). In this study, the effect of 200 µM acetosyringone on the production of H6 VLPs was investigated. Compared to no acetosyringone, the addition of 200 µM acetosyringone to the infiltration buffer appeared to slightly enhance the production of H6 HA_0 when the protein was subjected to immunodetection (Figure 2.5), although a definite increase in the presence of H6 VLP was apparent with TEM analysis. The concentration of acetosyringone was not increased beyond this point, as the H6 VLPs are intended for use as a H6 AI candidate vaccine for poultry and low production costs are a priority. Thus, the addition of 200 µM acetosyringone enhances the expression of H6 VLPs in *N. benthamiana*.

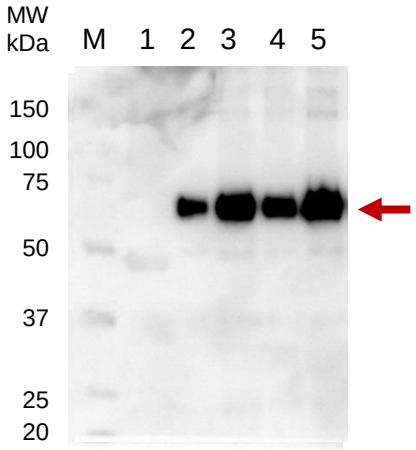


Figure 2.5: Immunodetection of partially-purified H6 hemagglutinin (HA_0), demonstrating the effect of the addition of acetosyringone on protein yield. Lane 1: Negative control (pEAQ-HT-empty); lanes 2 and 3: 0 µM acetosyringone, $H6_{HA}S1C$ present in Iodixanol fractions 9 and 10, respectively; lane 4 and 5: 200 µM acetosyringone, $H6_{HA}S1C$ present in Iodixanol fractions 9 and 10, respectively. M: WesternC protein molecular weight marker. The arrow indicates the position of the target protein (H6 HA_0) (approximately 62 kDa).

2.3.3. The effect of *Agrobacterium* culture density on protein yield

Determining the optimal *Agrobacterium* culture density is important to increase the efficiency of transient expression, as a low protein yield will be obtained if the concentration is too low, while a too high concentration could lead to necrosis due to a hypersensitive response in the infiltrated leaf tissue (Wroblewski *et al.,* 2005; Loh and Wayah, 2014). According to Norkunas (2014), increasing the culture density to an OD_{600} of 2 and 2.5 resulted in the highest expression of the target protein, although culture densities at 0.1, 0.5, 1.5 or 3 did not result in significantly increased expression levels in comparison to the control (OD_{600} = 1). In this study, *Agrobacterium* culture densities (OD_{600}) of 2, 1.5, 1 and 0.5 were compared and a clear difference in protein yield was not apparent between an OD_{600} of 2, 1.5 and 1, while an OD_{600} = 0.5 resulted in a noticeable reduction in protein expression (data not shown). With a culture density of 2, a mild necrotic response and discoloration (yellowing) of infiltrated leaves were prominent from 5 dpi onwards. Thus, for the production of H6 VLPs, a culture density of 1 resulted in efficient transient expression without signs of leaf necrosis.

2.4. Conclusions

Transient plant-based expression has been widely adopted to produce pharmaceutically important and commercially valuable proteins. Advanced vector technologies, host line development and enhanced *A. tumefaciens* transformation efficiencies have overcome previous issues of low protein yield, biocontainment, the limitation of transgene size, and non-mammalian glycosylation. However, the production methodology needs to be tailored to the target protein in order to maximize yield and ensure its commercial viability. Transient plant-based expression can be improved at various stages of the agroinfiltration process to maximize protein yield. In this study, agroinfiltration of *N. benthamiana* with the pEAQ-HT vector system (Sainsbury and Lomonossoff, 2008) was used to establish a platform for the production of H6 hemagglutinin-based influenza VLPs.

Codon optimization of the foreign gene to more closely reflect codon usage of the expression host is frequently used to improve expression levels, although the benefits of this process have been inconsistent and empirical. In this study, to investigate the effect of codon optimization on protein yield, a full-length hemagglutinin gene based on a 2016 sub-lineage I H6N2 virus isolated from chickens in South Africa was codon optimized for expression in chickens, humans and plants, respectively. Of these, only the chicken and human codon optimized variants yielded detectable levels of the target protein, possibly due to higher GC contents (51.52% and 56.32%, respectively) in comparison to the plant codon optimized

variant (36.86%) (de Rocher *et al.,* 1998; Suo *et al.,* 2006; Jiang *et al.,* 2007; Stachyra *et al.,* 2016).

Agrobacterium strains have variable virulence and transformation efficiencies (Hellens *et al.,* 2000; Norkunas, 2014). In order to compare the effect of the choice of *A. tumefaciens* strain on transient production of target protein yield, three laboratory strains, namely GV3101::pMP90, AGL-1 and LBA4404, were used to deliver the chicken and human codon optimized variants into the plant for replication. In addition, in order to determine if the co-expression with the influenza M2 ion channel protein could result in increased protein yield for the H6 subtype (Jutras *et al.,* 2015), each variant was also co-expressed with M2. For the chicken codon optimized variant, sub-lineage I H6 HA_0 was successfully produced using all three *A. tumefaciens* strains and the highest yield was obtained with AGL-1 and by co-expression with influenza M2 (HA titer of 512 (9 $\log_2$)). With human codon optimization, however, sub-lineage I H6 HA_0 were only successfully produced harnessing GV3101::pMP90 and LBA4404. The use of LBA4404 resulted in the highest yield (HA titer of 512 (9 $\log_2$)) and enhanced target protein expression was not obtained with influenza M2 co-expression. Given the difficulty of hand infiltration using LBA4404 at higher culture densities, the chicken codon optimized variant, *A. tumefaciens* strain AGL-1 and co-expression with M2 (plant codon optimized) were selected for subsequent optimization experiments. However, given the advantage of chicken (or human) codon optimization over plant codon optimization demonstrated in this study, whether co-expression with chicken codon optimized M2 will further augment the expression of H6 VLPs remains to be established in future.

The addition of acetosyringone, a chemical inducer of *Agrobacterium* virulence genes, as well as the density of the *Agrobacterium* culture introduced into the plant, has been shown to affect transient plant-based expression (Wydro *et al.,* 2006; Norkunas, 2014). Here, the addition of 200 µM acetosyringone to the infiltration buffer enhanced the production of H6 VLPs. Furthermore, a clear difference in protein yield was not apparent between an OD_{600} of 2, 1.5 and 1, although a mild necrotic response and yellowing of infiltrated leaves were prominent from 5 dpi onwards at an OD_{600} of 2. An OD_{600} of 0.5 resulted in a noticeable reduction in protein expression. Thus, a culture density of 1 to 1.5 was sufficient for the transient expression of sub-lineage I H6 VLPs in *N. benthamiana*.

Chapter 3

Optimization of influenza H6 VLP production in *N. benthamiana*: Protein extraction and purification

3.1. Introduction

Appropriate downstream processing (DSP) is critical for plant-produced target proteins. DSP of transient protein production in plants include protein extraction from the infiltrated leaf material, clarification of the plant extract and purification of the target protein. DSP accounts for a significant portion of the total operating costs for product manufacturing and the removal of contaminants is crucial in ensuring good manufacturing practice (GMP) compliance (Wilken and Nikolov, 2012; Lojewska *et al.,* 2016). Thus, keeping the DSP steps to a minimum is vital for the production of a low-cost product.

The extraction buffer plays an important role in protein stability by maintaining a constant pH in the solution. The isoelectric point (pI) of the target protein is usually an indication of the optimal pH for protein extraction, although the pH of the plant cell compartment where the product is produced also needs to be taken into consideration. A wide variety of biological buffers are available, each with a different pH range where its buffering capacity is at its most effective, and protease inhibitors are usually added to the extraction buffer to prevent proteolysis of the target protein via host proteases (Azzoni *et al.,* 2005; Grosse-Holz *et al.,* 2018). However, the interaction of some buffers with metals can affect the biological system (e.g. enzyme activity), and the choice of extraction buffer, its concentration, pH, salinity and additives (e.g. dithiotreitol (DTT), N-Lauroylsarcosine sodium salt (NLS) or sodium metabisulfite) can consequently impact protein yields and downstream processes (Azzoni *et al.,* 2005; Thuenemann, 2010; Majorek *et al.,* 2014; Ferreira *et al.,* 2015; Booth *et al.,* 2018). Buffers that have been used for the extraction of VLPs transiently produced in *Nicotiana benthamiana* include Tris (Tris(hydroxymethyl)aminomethane), Bicine and phosphate buffered saline (PBS) (Landry *et al.,* 2010; Thuenemann, 2010; Ruiz *et al.,* 2018; Mokoena *et al.,* 2019). PBS buffer is widely used as a placebo in clinical and animal trials. When buffers beside PBS are used for protein extraction, dialysis in PBS usually occurs prior to administration of the plant-based heterologous protein (Landry *et al.,* 2010; Thuenemann *et al.,* 2013).

The purification method is a major factor in heterologous protein expression and largely determines the cost of the product. The purification strategy is tailored for each individual protein based on inherent properties like solubility, size, charge, pI, and hydrophobicity and

should not contain more than three processing steps to maximize purification efficiency (Chen, 2008; Lojewska *et al.,* 2016). For human monoclonal antibodies and VLP vaccines, purification typically entail the combined use of membrane-based separation (i.e. ultrafiltration and diafiltration (e.g. tangential flow filtration (TFF)) and one or more column chromatography steps (e.g. size exclusion and ion exchange chromatography) to attain the purity required (Landry *et al.,* 2010; Lai and Chen, 2012; O'Hara *et al.,* 2012). For veterinary products, however, the purification requirements are a lot less stringent and the use of ultrafiltration/TFF for concentration and partial purification, as well as the use of minimally purified (i.e. clarified) plant extract, have been reported for VLP vaccines (Ruiz *et al.,* 2018; Mokoena *et al.,* 2019).

On a laboratory scale, the use of affinity-based chromatography techniques, immobilized metal affinity chromatography (IMAC) in particular, have also been extensively explored for the purification of pharmaceutical important and commercially valuable molecules (Zhao *et al.,* 2018). IMAC purification is based on the interaction between the affinity tag on the surface of the target molecule (particularly poly-histidine (His 6X)) with immobilized metal ions (e.g. Nickel (Ni^{2+}), Copper (Cu^{2+}), Cobalt (Co^{2+}), Zink (Zn^{2+}), or Iron (Fe^{3+})) with high binding specificity. IMAC purification of His-tagged protein is considered to be cost-effective and relatively simple with potential at commercial scale, and commercial antibodies against the His sequence could be employed for quantification and detection of the recombinant protein (Hu *et al.,* 1999; Kuo and Chase, 2011; Pereira *et al.,* 2012; Zhou *et al.,* 2018). IMAC could possibly reduce a multistep purification process to a single step, especially for applications where high sample purity is not required (i.e. for animal health products), which is a major advantage in terms of process time and production costs (Lichty *et al.,* 2005; Kuo and Chase, 2011; Lojewska *et al.,* 2016). However, the addition of the His-tag could potentially affect expression levels and protein folding, which could result in a non-functional protein or loss of conformational epitopes and consequently, reduced efficacy in the case of an immunogenic antigen (i.e. a vaccine or adjuvant) (Hu *et al.,* 1999; Kuo and Chase, 2011; Pereira *et al.,* 2012; Krupka *et al.,* 2016; Booth *et al.,* 2018; Zhou *et al.,* 2018). The position of the His-tag (e.g. N- or C-terminus) could also result in the tag being hidden or insufficiently exposed to facilitate effective binding to the immobilized metal ions, resulting in poor recovery of the target protein. Other factors like the adsorption matrix (e.g Tris-(carboxymethyl)-ethylenediamine (TED) or Iminodiacetic acid (IDA)) and buffer compositions could also affect the binding efficiency (https://www.mn-net.com/tabid/1450/default.aspx). Poly-His-tagged VLPs that are efficacious have been produced in bacteria (*Escherichia coli*), yeast and insect cells, and a His-tagged bluetongue virus VP2 capsid protein, which can assemble with other

bluetongue virus structural proteins to form VLPs, has been successfully produced in *N. benthamiana* and purified by IMAC (Hu *et al.,* 1999; Pereira *et al.,* 2012; Thuenemann *et al.,* 2013; Koho *et al.,* 2015; Manuel-Cabrera *et al.,* 2016; Bustos-James *et al.,* 2017; Fay *et al.,* 2019). Thus, the feasibility of using IMAC purification is protein dependent and the position of the His-tag is critical.

This chapter describes the optimization of DSP for H6 sub-lineage I and II influenza VLPs transiently produced in *N. benthamiana.* By making use of the sub-lineage I and II chicken codon optimized constructs, the effect of different extraction buffers (namely Tris, Bicine and PBS) on protein yield was determined by making use of differential ultracentrifugation as purification method. In addition, the feasibility of IMAC purification was investigated by incorporating a poly-His-tag at the N- or C-terminus of the HA protein. The effect of the addition of a His-tag on protein expression levels and the intactness of the assembled H6 influenza VLP was assessed by making use of differential centrifugation as purification method, while the efficiency of IMAC purification of the His-tagged proteins was investigated by making use of Ni-TED or Ni-IDA resins. Optimization of these factors are important to ensure the most cost-effective manufacturing possible and will contribute to the establishment of a production platform for H6 influenza VLPs in *N. benthamiana*.

3.2. Materials and methods

3.2.1. Ethical considerations

As discussed in section 2.2.1 (Appendix A).

3.2.2. Construction and validation of expression vectors used for the optimization of protein extraction and purification

3.2.2.1. Gene design and synthesis

Based on the codon optimization results obtained for the sub-lineage I H6 synthetic gene (discussed in Chapter 2 section 2.3.1), a synthetic gene was designed based on the hemagglutinin (HA) gene sequence of a sub-lineage II LPAI H6N2 AI virus (A/chicken/South Africa/BKR4/2012; GenBank: KX595260.1) and codon optimized by Bio Basic Canada Inc. for expression in chickens (*Gallus gallus*) (Appendix B). The native signal peptide sequence was replaced by a sequence encoding the *Mus musculus* monoclonal antibody heavy chain variable region signal peptide (O'Hara *et al.,* 2012) and *Age*I and *Xho*I restriction enzyme recognition sites were incorporated at the 5'- and 3'-terminals, respectively, for cloning into pEAQ-HT (Figure 3.1). In this chapter, $H6_{HA}$S1C and $H6_{HA}$S2C refer to the synthetic chicken codon optimized genes based on the HA of sub-lineage and II H6N2 viruses, respectively.

3.2.2.2. Primer design for PCR amplification of H6 HA_0 DNA sequences with a His-tag

To investigate the feasibility of using IMAC purification for H6 VLPs, a poly (6X)-His-tag was incorporated at the N- or C-terminus of the synthetic genes using polymerase chain reaction (PCR) primers. The PCR primers (Inqaba Biotec™) were designed so that the resulting fragments contain the murine signal peptide sequence and appropriate restriction enzyme recognition sites (Figure 3.1). The C-terminal His-tag PCR primer was designed to introduce the poly-His-tag at the C-terminal end of the H6 HA_0 gene, followed by the termination codon sequence and the required restriction enzyme recognition site (*Xho*I) at the 5'end. Using the $H6_{HA}$S1C synthetic gene as template, PCR was performed to incorporate a N-terminal poly-His-tag sequence ($H6_{HA}$S1C-N_{His}), a C-terminal poly-His-tag sequence ($H6_{HA}$S1C-C_{His}), or a N-terminal poly-His-tag sequence followed by a linker sequence (encoding 4 x Glycine (G) and 1 x Serine (S) residues) ($H6_{HA}$S1C-N_{His}-L). Using the $H6_{HA}$S2C synthetic gene as template, PCR was performed to incorporate a N-terminal poly-His-tag sequence ($H6_{HA}$S2C-N_{His}). In addition, in order to determine if the incorporation of the Kozak consensus sequence would enhance protein expression (Kozak, 1987; Kanagarajan *et al.,* 2012), PCR was used to add the Kozak plant translation initiation sequence (GCCACC) immediately upstream of the ATG initiation codon of the $H6_{HA}$S1C-N_{His} and $H6_{HA}$S2C-N_{His} inserts, yielding $H6_{HA}$S1C-N_{His}-Kz and $H6_{HA}$S2C-N_{His}-Kz, respectively.

The PCR reactions were performed using the KAPA HiFi™ PCR kit (KAPA Biosystems), which contains a high-fidelity polymerase and proofreading to ensure superior accuracy during amplification of the gene of interest. The PCR reactions were performed in a Mastercycler® EP gradient S thermocycler (Eppendorf) and consisted of the following: 5 µl of 5 X KAPA HiFi buffer, 0.75 µl dNTP mix, 0.2 µM forward and reverse primer, respectively (Table 3.1), 0.5 µl of KAPA HiFi DNA polymerase (5 U/µl), template DNA, and sterile nuclease-free water to a final volume of 25 µl. With the exception of $H6_{HA}$S1C-N_{His}-L and $H6_{HA}$S2C-$N_{His,}$ the cycling conditions were as follows: 1 cycle at 95°C for 2 minutes, 35 cycles of 98°C for 20 seconds, the appropriate annealing temperature (T_a) for 30 seconds (Table 3.1), 72°C for 30 seconds, with a final extension step of 72°C for 5 minutes. For $H6_{HA}$S1C-N_{His}-L and $H6_{HA}$S2C-N_{His}, the cycling conditions were modified to increase amplification of the target product and included the following: 1 cycle at 95°C for 2 minutes, 10 cycles of 98°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by 25 cycles of 98°C for 20 seconds, the appropriate T_a (Table 3.1) for 45 seconds, 72°C for 30 seconds and a final extension step of 72°C for 5 minutes. PCR products were resolved on a 1% [w/v] agarose gel, and the amplicons were excised from the gel and purified using the Zymoclean gel DNA recovery kit (Zymo Research), according to manufacturer's instructions.

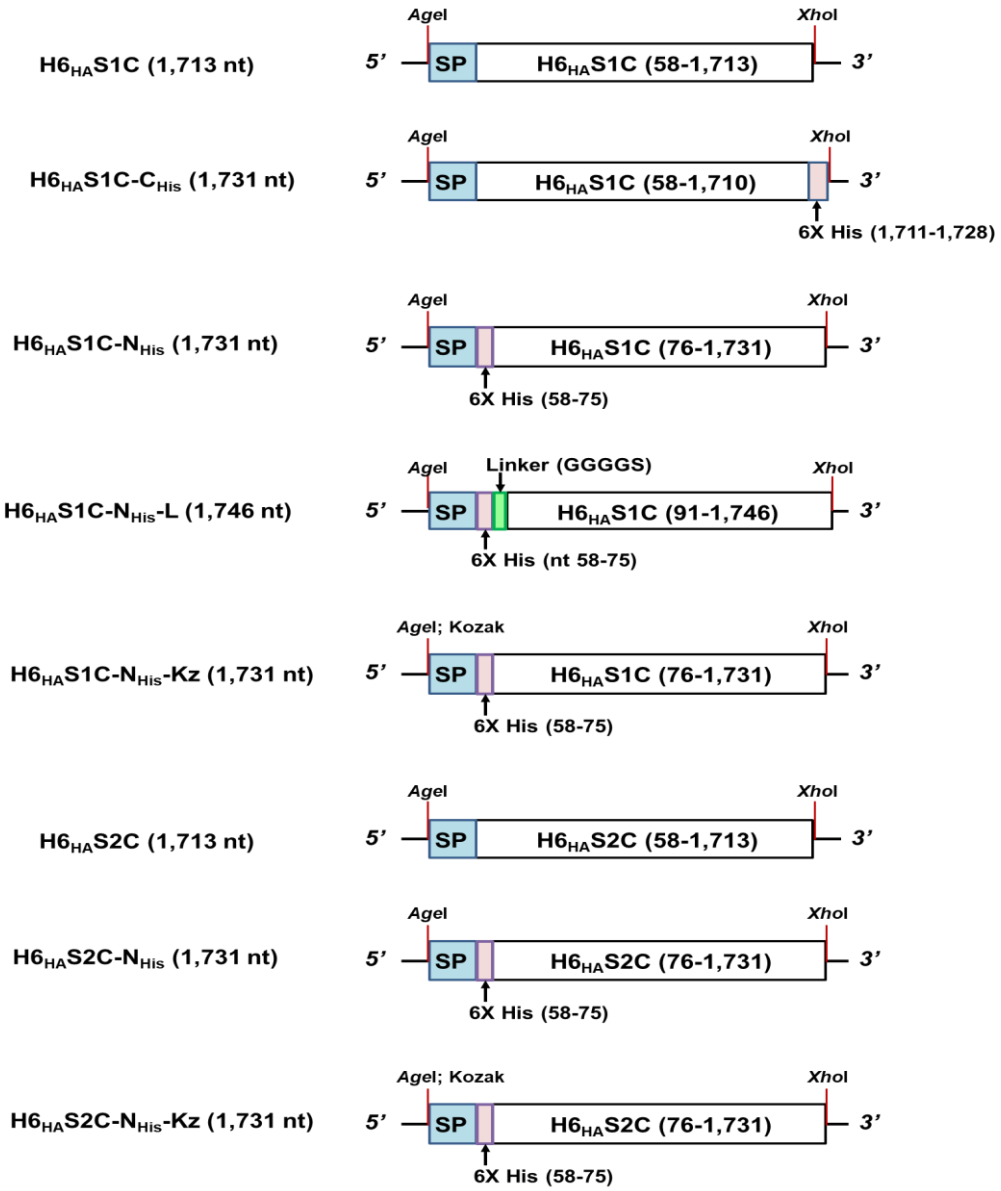


Figure 3.1. Schematic representation of the H6 gene inserts used in this chapter. For the sub-lineage I chicken codon optimized H6 hemagglutinin (HA_0) gene ($H6_{HA}S1C$), a 6x His-tag sequence was incorporated at the C-terminus ($H6_{HA}S1C\text{-}C_{His}$) and N-terminus ($H6_{HA}S1C\text{-}N_{His}$), a linker sequence encoding four glycine (G) residues and one serine (S) residue was incorporated between the N-terminal 6X His-tag and the H6 HA_0 gene ($H6_{HA}S1C\text{-}N_{His}\text{-}L$), and the Kozak consensus sequence was included immediately upstream of the start codon (after the *Age*I restriction enzyme recognition site) ($H6_{HA}S1C\text{-}N_{His}\text{-}Kz$). For the sub-lineage II chicken codon optimized H6 HA_0 gene ($H6_{HA}S2C$), a 6x His-tag sequence was incorporated at the N-terminus ($H6_{HA}S2C\text{-}N_{His}$) and the Kozak consensus sequence was included immediately upstream of the start codon. The murine signal peptide (SP) sequence was incorporated at the N-terminus and *Age*I and *Xho*I restriction enzyme recognition sites at the N- and C-termini, respectively, of each gene. For each insert, the nucleotide (nt) numbering (as determined from the start codon to the termination codon) is provided in brackets.

Table 3.1: Sequences of the primers used in this study to generate histidine (His)-tagged H6 hemagglutinin (HA_0). For each of the primer sets, the annealing temperature (T_a) is provided.

Insert	Primer sequence (5' to 3')	T_a (°C)
$H6_{HA}$S1C-N_{His}	F:TTAACCGGTATGGGATGGAGCTGGATCTTTCTTTTCCTCCTGTCAGGAGCTGCAGGTGTCCATTGCCACCACCACCACCACCATGATAAGATCTGCAT	68
	R:TCCCTCGAGTTAGATGCACACTCTGCACTGC	
$H6_{HA}$S1C-C_{His}	F:TTAACCGGTCCATGGGATGGTCCTGG	68
	R:TCCCTCGAGTTAATGGTGGTGGTGGTGGTGGATGCACACTCTGC	
$H6_{HA}$S1C-N_{His}-L	F:TTAACCGGTATGGGATGGAGCTGGATCTTTCTTTTCCTCCTGTCAGGAGCTGCAGGTGTCCATTGCCACCACCACCACCACCATGGCGGGGGCGGGAGCGATAAGATCTGCAT	68
	R:TCCCTCGAGTTAGATGCACACTCTGCACTGC	
$H6_{HA}$S1C-N_{His}-Kz	F: TTAACCGGTGCCACCATGGGATGGAGCTG	66
	R: TCCCTCGAGTTAGATGCACACTCTGCACTGC	
$H6_{HA}$S2C-N_{His}	F: TTAACCGGTATGGGATGGAGCTGGATCTTTCTTTTCCTCCTGTCAGGAGCTGCAGGTGTCCATTGCCACCACCACCACCACCATGACAAGATCTGCAT	64
	R: TCCCTCGAGTCAGATGCAGATTCTGCAC	
$H6_{HA}$S2C-N_{His}-Kz	F: TTAACCGGTGCCACCATGGGATGGAGCTG	63
	R: TCCCTCGAGTCAGATGCAGATTCTGCAC	

$H6_{HA}$S1C-N_{His}: sub-lineage I chicken codon optimized H6 HA_0 gene insert with a N-terminal 6 x His-tag; $H6_{HA}$S1C-C_{His}: sub-lineage I chicken codon optimized H6 HA_0 gene insert with a C-terminal 6 x His-tag; $H6_{HA}$S1C-N_{His}-L: sub-lineage I chicken codon optimized H6 HA_0 gene insert with a N-terminal 6 x His-tag followed by a linker sequence; $H6_{HA}$S1C-N_{His}-Kz: sub-lineage I chicken codon optimized H6 HA_0 gene insert with a N-terminal 6 x His-tag followed by the Kozak consensus sequence; $H6_{HA}$S2C-N_{His}: sub-lineage II chicken codon optimized H6 HA_0 gene insert with a N-terminal 6 x His-tag; $H6_{HA}$S2C-N_{His}-Kz: sub-lineage II chicken codon optimized H6 HA_0 gene insert with a N-terminal 6 x His-tag, followed by the Kozak consensus sequence; F: Forward primer; R: Reverse primer.

3.2.2.3. Cloning of H6 HA_0 gene fragments into the pEAQ-HT plant expression vector and transformation of competent DH10B cells

The synthetic chicken codon optimized sub-lineage II H6 gene (Bio Basic Canada Inc.) was excised from the carrier vector (pUC57) by restriction enzyme digestion (*Age*I/*Xho*I) and cloned into pEAQ-HT as described in Chapter 2, section 2.2.2.2. For the cloning of the His-tagged inserts (Table 3.1.) into pEAQ-HT, the respective purified PCR products were digested with *Age*I and *Xho*I, purified using the Zymoclean gel DNA recovery kit (Zymo Research) and subsequently ligated into the pEAQ-HT expression vector previously digested with *Age*I and *Xho*I using the Fast-Link DNA Ligase kit (Epicentre) according to the manufacturers' instructions. The ligation reaction mixtures were subsequently transformed into competent *Escherichia coli* (*E. coli)* DH10B cells and bacterial clones resistant to antibiotics were selected for verification via colony PCR and Sanger DNA sequencing, as described previously (Chapter 2, section 2.2.2.3.).

3.2.3. Transformation of competent *Agrobacterium tumefaciens* (AGL-1) and introduction into *N. benthamiana*

Following selection of positive recombinant clones from section 3.2.2.3, each of the plasmids was transformed into competent *A. tumefaciens* AGL-1 cells and antibiotic-resistant clones were selected for verification via colony PCR, as described previously (Chapter 2, section 2.2.3.). Each of the constructs were prepared for infiltration as described previously (Chapter 2, section 2.2.4.), with the final *Agrobacterium* suspension (OD_{600} = 1.5, unless stated otherwise) comprising of equal parts pEAQ-HT+M2 and pEAQ-HT+$H6_{HA}$. After the *A. tumefaciens* suspensions were incubated at room temperature for at least an hour, a syringe without a needle (unless stated otherwise) was used to introduce the respective bacterial suspensions into mature leaves of *N. benthamiana* that have been modified to exclude plant-specific glycosylation (Strasser *et al.,* 2008).

In one experiment, vacuum infiltration was tested in combination with depth filtration/TFF to provide preliminary results for larger scale production and purification. For vacuum infiltration, *Agrobacterium* suspensions of OD_{600} = 0.4, comprising of equal parts pEAQ-HT+M2 and pEAQ-HT+$H6_{HA}$S1-N_{His}, were introduced into *N. benthamiana* leaves under a vacuum of 30 mbar, with a 4 minute holding time (Memmer incubator connected to a vacuum pump; all automated). A total of 24 pots (48 plants) were vacuum infiltrated, six pots at a time, and one pot was hand infiltrated to serve as a control.

3.2.4. Protein extraction, clarification and purification

At day 6 post infiltration (dpi) (unless otherwise indicated) the total soluble protein (TSP) was extracted from the infiltrated leaves by homogenization in two volumes of cold extraction buffer supplemented with proteinase inhibitor cocktail (P2714, Sigma-Aldrich) immediately prior to extraction to prevent proteolysis of the target protein. Three buffers that have previously been used to extract VLPs were tested in an effort to identify an appropriate buffer for the extraction of H6 influenza VLPs produced *N. benthamiana*: Tris (50 mM Tris, 150 mM sodium chloride (NaCl), and 0.04% [w/v] sodium metabisulfite, pH 8.0), Bicine (50 mM Bicine, 20 mM NaCl and 0.1% [w/v] NLS sodium salt, pH 8.4), and PBS (4.3 mM sodium phosphate ($NaHPO_4$), 1.4 mM monopotassium phosphate (KH_2PO_4), 2.7 mM potassium chloride (KCl), and 127 mM NaCl, pH 7.4) supplemented with 0.04% [w/v] sodium metabisulfite (Landry *et al.,* 2010; Thuenemann *et al.,* 2013; Ruiz *et al.,* 2018). The extract was clarified as described previously (Chapter 2, section 2.2.4.1.) and subjected to purification.

The target protein was purified using differential centrifugation (i.e. iodixanol density gradient (20-60%)) as described previously (Chapter 2, section 2.2.4.1.) and/or immobilized metal ion affinity chromatography (IMAC) (His-tagged H6 protein) with nickel as metal ion, according to the manufacturer's instructions. To confirm that the His-tagged target proteins still assemble to form VLPs, the plant extract was subjected to density gradient centrifugation followed by transmission electron microscopy (TEM) analysis. To investigate the suitability of IMAC purification for H6 VLPs, equilibrated Protino® Ni-TED (Tris-carboxymethyl ethylene diamine) pre-packed 2000 columns (Macherey-Nagel) were used to purify 5 or 10 ml clarified plant extract, according to manufacturer's instructions with the buffer solutions provided. Bound protein was eluted with imidazole-containing buffer and at least three 500 µl elution fractions were collected. The flow through, wash and elution fractions were subjected to biochemical analysis to determine the presence of the target protein. To determine the effect of different resins on the purification efficiency, Protino® Ni-TED and Protino® Ni-IDA resins (Macherey-Nagel) were directly compared, with buffer solutions prepared as specified by the manufacturer. Protino® Ni-TED and Protino® Ni-IDA columns with a 5ml bed volume (2.5 milligrams (mg) resin) were prepared according to manufacturer's instructions, whereafter 10 ml of clarified plant extract was added to the equilibrated columns for purification. Bound protein was eluted with imidazole-containing buffer, with at least three 500 µl elution fractions collected for analysis alongside the preceding fractions (i.e. flow through and wash fractions). To determine whether column overloading contributed to loss of protein in the preceding fractions, a larger scale purification of histidine-tagged sub-lineage I H6 VLPs (310 ml of clarified plant extract) was subsequently conducted on the ÄKTA™ Avant 150 (GE

Healthcare Life Sciences) operated via Unicorn 6 software. The column used was a 7 ml bed volume Protino® Ni-TED resin packed in an XK16 column. The parameters included the following: a flow rate of 2 ml/minute, 3 column volumes (CV) of equilibration buffer, 5 CV of wash buffer, and 7 CV of imidazole-containing elution buffer. The eluate was collected in 5 ml fractions for analysis alongside the preceding fractions.

A commercially scalable membrane-based purification method that is widely used for the rapid and efficient clarification, concentration, and purification of proteins, namely depth filtration followed by ultrafiltration (TFF), was investigated for the N-His-tagged sub-lineage I protein ($H6_{HA}$S1C-N_{His}). Clarified plant extract (1.1 L) (extracted in Tris buffer), was subjected to depth filtration (Sartoclean GF Sterile MidiCap, Sarotrius Stedim Biotech, Göttingen, Germany), whereafter approximately 900 ml of filtered extract was subjected to TFF using the Minimate™ TFF capsule with Omega 300K membrane (PALL Life Sciences), with the pressure not exceeding 2 bar. The filtrate was washed with 600 ml of PBS buffer, with a final concentrated volume of approximately 67 ml. Dilutions of TFF (1 in 2, 1 in 4 and 1 in 10) was subjected to density gradient centrifugation alongside the hand infiltrated extract that served as a control, followed by BCA quantification of selected iodixanol fractions to estimate the yield per kilogram (kg) leaf material.

3.2.5. Biochemical analysis and confirmation of identity

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), TEM, hemagglutination assay and liquid chromatography-mass spectrometry (LC-MS/MS)-based peptide sequencing (for the sub-lineage II HA protein ($H6_{HA}$S2C), Appendix C) were conducted as described previously (Chapter 2, section 2.2.5). Immunoblotting was also performed as described previously, with one modification: IMAC purified proteins were detected with mouse anti-His horseradish peroxidase (HRP) conjugated antibody (1:1,500; Sigma) and/or avian influenza H6N2 antiserum (1:600 dilution; Deltamune) in conjunction with goat anti-chicken IgY HRP conjugated antibody (1:1,500; Novex Life Technologies, A16054). Differential centrifugation purified proteins were detected with avian influenza H6N2 antiserum in conjunction with goat anti-chicken IgY HRP conjugated antibody, as described previously.

The Pierce™ Micro BCA protein assay kit (Thermo Fisher Scientific ™) was used to estimate the protein yield obtained following vacuum infiltration and TFF purification. BCA quantification was performed for each of the TFF samples (1 in 2, 1 in 4 and 1 in 10 dilutions) subjected to density gradient centrifugation in order to estimate the total H6 HA_0 protein content. For each TFF dilution samples, as well as the hand infiltrated control sample, iodixanol fraction 10 was selected for BCA quantification as it contained less contaminating proteins in relation to the target protein compared to iodixanol fractions 11 or 12 (as assessed by SDS-PAGE analysis, data not shown). A serial dilution (1 in 10, 1 in 40 and 1 in 80) was prepared for each sample to be quantified. Standards (Albumin standard (BSA)) ranging from 1 to 100 µg per ml were prepared according to manufacturer's instructions and protein quantification was performed in triplicate, as recommended (absorbance measured at 562 nm), with nuclease-free water serving as negative control samples. A standard curve was generated from the BSA standards and used to estimate the protein yield per kg leaf material.

3.3. Results and discussion

3.3.1. The effect of the extraction buffer on protein yield

The extraction buffer plays a critical role in the stability of the protein by maintaining a constant pH over a given range (Azzoni *et al.,* 2005; Lojewska *et al.,* 2016). Different buffers have been reported for the extraction of transiently expressed VLPs in *N. benthamiana*, including a Tris buffer for influenza VLPs (Landry *et al.,* 2010), a Bicine buffer for bluetongue VLPs (Thuenemann *et al.,* 2013) and PBS for foot-and-mouth disease VLPs (Ruiz *et al.,* 2018). In this study, these buffer solutions were compared for the extraction of transiently expressed H6 VLPs. For the Bicine buffer, however, DTT was excluded from the solution reported by Thuenemann *et al.,* (2013) for this study, as DTT would break the disulphide bond linking the two influenza hemagglutinin subunits (HA_1 and HA_2) (Webster *et al.,* 1992).

3.3.1.1. Tris vs. Bicine as extraction buffer

Making use of the pEAQ-HT+$H6_{HA}$S1C construct (AGL-1; co-expressed with M2), the Tris and Bicine buffer solutions supplemented with protease inhibitors were used to extract the target protein at different time points post infiltration (dpi), followed by density gradient centrifugation. Based on SDS-PAGE analysis and Immunodetection of the partially-purified samples (fraction 10) a clear difference in yield between the two buffers was not apparent. However, the use of Bicine appear to result in slightly higher yields of H6 HA_0 (Figure 3.2). The expression levels appeared to be at its highest at 6 dpi using Tris, while the yield appeared to be at its highest at 4 dpi with Bicine (Figure 3.2). With TEM analysis, however, H6 VLPs were only observed for the Tris samples from day 5 onwards, with the largest

number of VLPs present at 6 dpi. Increasing the NaCl concentration of the Bicine buffer solution from 20 to 150 mM yielded high levels of H6 HA_0, but TEM analysis revealed a lack of intact VLPs (data not shown). Thus, the Tris buffer was considered to be more suited for the extraction of plant-produced H6 influenza VLPs than Bicine buffer.

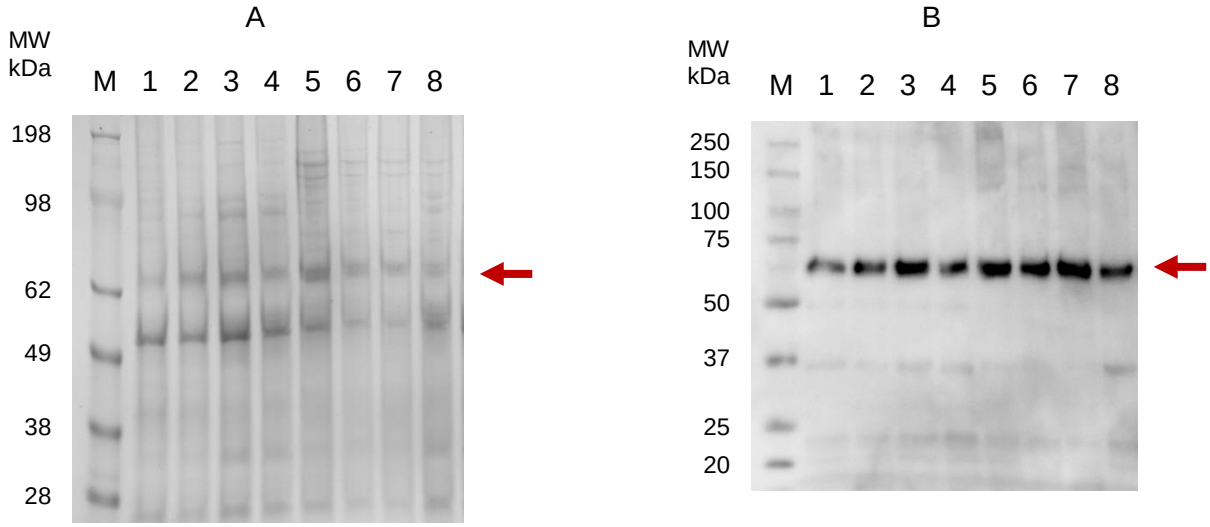


Figure 3.2. SDS-PAGE (A) and immunodetection (B) of sub-lineage I H6 hemagglutinin (HA_0) ($H6_{HA}$S1C), extracted using Tris or Bicine buffer at different days post infiltration (dpi). Lanes 1 to 4: $H6_{HA}$S1C extracted using Tris buffer at 4, 5, 6, or 7 dpi, respectively; Lanes 5 to 8: $H6_{HA}$S1C extracted using Bicine buffer at 4, 5, 6, or 7 dpi, respectively. M: The SeeBlue Plus 2 (A) and WesternC (B) protein molecular weight markers were used for the SDS-PAGE and immunoblot, respectively. The arrows indicate the position of the target protein (approximately 62 kDa). Tris: Tris(hydroxymethyl)aminomethane.

3.3.1.2. Tris vs. PBS as extraction buffer

PBS is widely used as a placebo in clinical and animal trials and dialysis in PBS prior to administration of the heterologous protein into the target host is common practice when an alternative extraction buffer is used (Landry *et al.,* 2010; Thuenemann *et al.,* 2013). As such, extraction in PBS would save time and consequently costs, which is especially important for poultry products. Making use of the pEAQ-HT+$H6_{HA}$S1C construct (AGL-1; co-expressed with M2) again, Tris buffer and PBS buffer containing sodium metabisulfite and protease inhibitors were used to extract the target protein (at 6 dpi), which were subsequently purified by differential centrifugation. SDS-PAGE and immunodetection of iodixanol fractions 10 and 11 revealed similar expression levels of H6 HA (Figure 3.3). Using TEM, intact VLPs were detected in fraction 10 for both buffers, with a slight increase in VLPs noted for PBS in

comparison to Tris. Fraction 10 purified samples were also tested for hemagglutination activity, yielding titers of 9 log_2 (512) and 10 log_2 (1024) for Tris and PBS, respectively, which corresponds with the results obtained with TEM. Thus, extraction with PBS buffer containing sodium metabisulfite resulted in the extraction of intact functional H6 influenza VLPs with protein yields comparable to that of Tris. The efficiency of PBS and Tris was also tested for other H6 HA_0 proteins and will be discussed intermittently in this chapter.

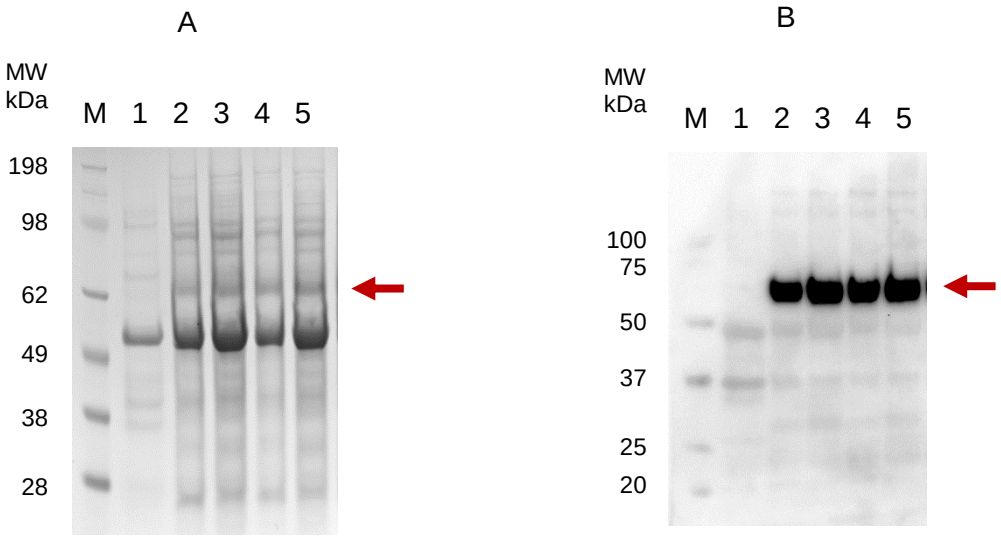


Figure 3.3. SDS-PAGE (A) and immunodetection (B) of partially-purified sub-lineage I H6 hemagglutinin (HA_0) ($H6_{HA}S1C$) extracted using Tris or PBS buffer. Lane 1: negative control (pEAQ-HT-empty); lanes 2 and 3: Tris extraction buffer, $H6_{HA}S1C$ present in Iodixanol fractions 9 and 10, respectively; lanes 4 and 5: PBS extraction buffer, $H6_{HA}S1C$ present in Iodixanol fractions 9 and 10, respectively. M: The SeeBlue Plus 2 (A) and WesternC (B) protein molecular weight markers were used for the SDS-PAGE and immunoblot, respectively. The arrows indicate the position of the target protein (approximately 62 kDa). Tris: Tris(hydroxymethyl)aminomethane; PBS: Phosphate buffered saline.

3.3.2. Protein purification via IMAC

IMAC is one of the methods commonly employed for the purification of recombinant protein, including VLPs (Kuo and Chase, 2011; Zhou *et al.,* 2018). The interaction between immobilized metal ions (particularly Ni^{2+}) on a matrix and the side chains of certain amino acids (particularly histidines) on the surface of the target protein enables purification, whereafter the purified target molecule is recovered from the matrix by elution with either low pH or imidazole-containing buffer. IMAC purification of His-tagged protein is considered to be cost-effective and relatively simple with potential at commercial scale, although the

position and length of the His-tag could potentially affect the expression levels, structure, stability, and function of the target protein, as well as its capacity to bind to immobilized metal ions for efficient purification (Hu *et al.,* 1999; Kuo and Chase, 2011; Park *et al.,* 2015; Booth *et al.,* 2018). Poly-His-tagged VLPs that are efficacious have been produced in bacteria (*E. coli*), yeast and insect cells, and the incorporation of the His-tag (primarily at the C-terminus of the capsid protein) resulted in enhanced expression levels in some cases (Hu *et al.,* 1999; Pereira *et al.,* 2012; Koho *et al.,* 2015; Manuel-Cabrera *et al.,* 2016; Bustos-James *et al.,* 2017). Other factors like the adsorption matrix (e.g Tris-(carboxymethyl)-ethylenediamine (TED) or Iminodiacetic acid (IDA)) and buffer compositions could also affect the binding efficiency (https://www.mn-net.com/tabid/1450/default.aspx). Thus, tailoring IMAC purification for the specific target protein is required to maximize protein yield and recovery of purified protein, as well as ensuring the immunogenicity of vaccine antigens like VLPs.

3.3.2.1. The effect of the addition of His-tag on the expression levels and assembly of VLPs

To determine whether the addition of a His-tag would lead to the formation of functional influenza H6 VLPs, a 6 x His-tag was added to the N- terminus ($H6_{HA}$S1C-N_{His}) and C-terminus ($H6_{HA}$S1C-C_{His}) of the sub-lineage I chicken codon optimized synthetic gene, respectively. The clarified plant extract was purified by differential centrifugation to demonstrate the formation of intact functional VLPs. SDS-PAGE and immunodetection of the partially-purified protein revealed that only the N-His-tagged construct resulted in the expression of H6 HA_0 (Figures 3.4 and 3.5) and the assembly of N-His-tagged protein into intact functional VLPs was confirmed by TEM analysis (Figure 3.6) and hemagglutination assay analysis. It is suspected that the addition of the poly-His-tag at the C-terminal resulted in steric hindrance that prevented the assembly of H6 HA trimers, and subsequently the formation of H6 VLPs. Interestingly, the addition of a His-tag at the N-terminus of sub-lineage I chicken codon optimized gene resulted in moderately higher protein expression levels (Figure 3.5), which also translated into an increase in the quantity of functional VLPs in comparison to the untagged version. Increased yields of recombinant proteins (including VLPs) following the addition of a His-tag have been reported previously with *E. coli* as expression system and, depending on the specific target protein, the presence of a N-His-tag could have either a negative or positive effect on protein stability (Svenson *et al.,* 2006; Park *et al.,* 2015; Manuel-Cabrera *et al.,* 2016; Booth *et al.,* 2018). In addition, the enhanced expression of sub-lineage I H6 HA_0 was accompanied by a slight decline in the accumulation of the chloroplast protein Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) (Figures 3.4 and 3.5), the most abundant soluble protein in plant leaves which consists of a

large subunit (approximately 55 kDa) and a small subunit (approximately 15 kDa) (Ma *et al.,* 2009; Oey *et al.,* 2009). A decline in Rubisco expression with high plant-based protein expression has been reported previously (Oey *et al.,* 2009).

The sub-lineage II chicken codon optimized construct (pEAQ-HT+H6$_{HA}$S2C) consistently yielded low expression levels with VLPs nearly completely undetected with TEM. Given the results obtained with the sub-lineage I N-His-tagged construct (pEAQ-HT+H6$_{HA}$S1C-NHis), a poly-His-tag was subsequently incorporated at the N-terminus of the sub-lineage II chicken codon optimized gene in an attempt to increase expression levels. Concurrently, the effect of the Kozak consensus sequence, which is frequently incorporated immediately upstream of the start codon to enhance the translational efficiency (Kozak, 1987; Kanagarajan *et al.,* 2012), on the expression of H6 HA$_0$ was tested using sub-lineage I and II N-His-tagged constructs (pEAQ-HT+H6$_{HA}$S1C-N$_{His}$ and pEAQ-HT+H6$_{HA}$S2C-N$_{His}$, respectively). In comparison to the untagged version, expression of the sub-lineage II H6 HA$_0$ protein was greatly enhanced with the addition of the N-His-tag (Figure 3.5) (with a corresponding reduction in the accumulation of Rubisco), a dramatic increase in the presence of influenza VLPs was observed with TEM (Figure 3.6), and an increase in hemagglutination titer from 5 $\log_2$ to 8 $\log_2$ was achieved. The addition of the Kozak sequence, however, resulted in a slight decline in protein yield for both the sub-lineage I and II constructs (Figure 3.5), with a reduction in hemagglutination titer from 10 $\log_2$ to 9 $\log_2$ and from 8 $\log_2$ to 7 $\log_2$, respectively. However, variations of the Kozak sequence are described in literature, with individual nucleotides upstream and downstream of the translation initiation sequence of variable importance (Kozak, 1987; Sawant *et al.,* 1999). In this study, the Kozak sequence described by Kanagarajan *et al.* (2012) was tested and was not successful in enhancing protein expression. However, it is not to say that an alternative Kozak sequence could not successfully enhance the expression of the target protein. Thus, for both the sub-lineage I and II chicken codon optimized genes, the addition of a N-His-tag enhanced protein expression levels in *N. benthamiana* while maintaining correct protein folding and activity of H6 VLPs that have been partially-purified by differential centrifugation.

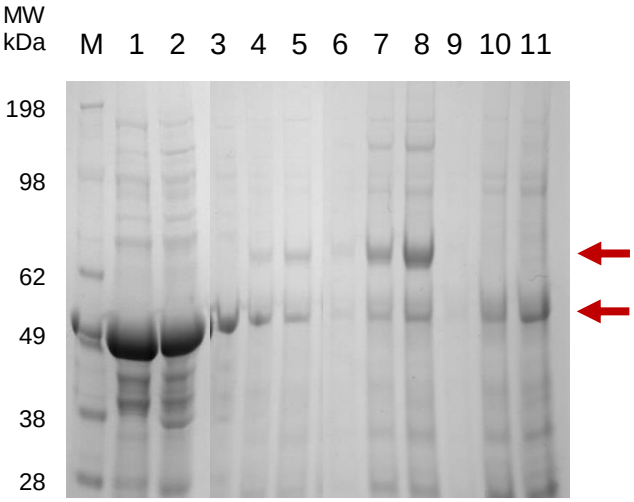


Figure 3.4. A composite SDS-PAGE gel image demonstrating the effect of the addition of a histidine (His)-tag at the N-terminal ($H6_{HA}S1C\text{-}N_{His}$) or C-terminal ($H6_{HA}S1C\text{-}C_{His}$) of sub-lineage I H6 hemagglutinin (HA_0) ($H6_{HA}S1C$) on protein yield. Proteins were extracted with Tris buffer and purified using differential centrifugation. Lane 1: negative control (un-infiltrated plant extract); lane 2: negative control (pEAQ-HT-empty); Lanes 3-5: $H6_{HA}S1C$, iodixanol fractions 9, 10 and 11; Lanes 6 to 8: $H6_{HA}S1C\text{-}N_{His}$, iodixanol fractions 9, 10 and 11; Lanes 9 to 11: $H6_{HA}S1C\text{-}C_{His}$, iodixanol fractions 9, 10 and 11. M: SeeBlue Plus 2 protein molecular weight marker. The arrows indicates the position of the target protein (approximately 63 kDa) and the large subunit of Rubisco (approximately 55 kDa). Tris: Tris(hydroxymethyl)aminomethane.

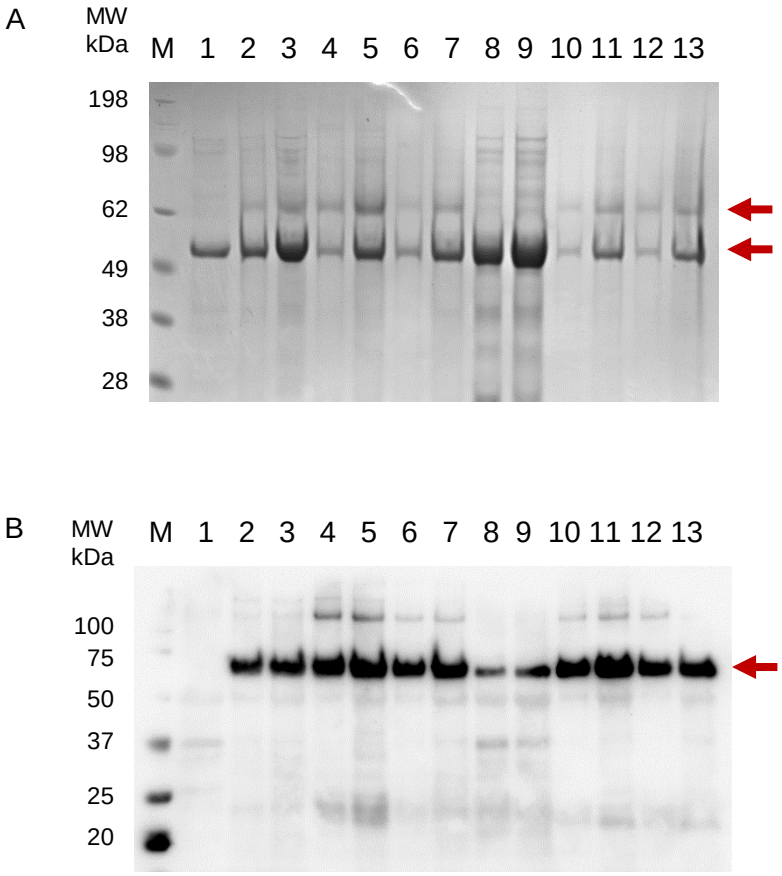


Figure 3.5. SDS-PAGE (A) and immunodetection (B) images demonstrating the difference in yield obtained with the addition of a N-terminal histidine (His)-tag and incorporation of the Kozak (Kz) sequence to the sub-lineage I and II H6 hemagglutinin (HA_0) gene inserts. Sub-lineage I H6 HA_0 proteins ($H6_{HA}S1C$, $H6_{HA}S1C\text{-}N_{His}$ and $H6_{HA}S1C\text{-}N_{His}$-Kz) and sub-lineage II H6 HA_0 proteins ($H6_{HA}S2C$, $H6_{HA}S2C\text{-}N_{His}$ and $H6_{HA}S2C\text{-}N_{His}$-Kz) were extracted with phosphate buffered saline (PBS) buffer and purified using differential centrifugation. Lane 1: negative control (pEAQ-HT-empty), Lanes 2 & 3: $H6_{HA}S1C$, present in Iodixanol fractions 10 & 11; Lanes 4 & 5: $H6_{HA}S1C\text{-}N_{His}$, present in Iodixanol fractions 10 & 11; Lanes 6 & 7: $H6_{HA}S1C\text{-}N_{His}$-Kz, present in Iodixanol fractions 10 & 11; Lanes 8 & 9: $H6_{HA}S2C$, present in Iodixanol fractions 10 & 11; Lanes 10 & 11: $H6_{HA}S2C\text{-}N_{His}$, present in Iodixanol fractions 10 & 11; Lanes 12 & 13: $H6_{HA}S1C\text{-}N_{His}$-Kz, present in Iodixanol fractions 10 & 11. M: The SeeBlue Plus 2 (A) and WesternC (B) protein molecular weight markers were used for the SDS-PAGE and immunoblot, respectively. The arrows indicate the position of the N-His-tagged H6 HA_0 protein (approximately 63 kDa) and the large subunit of Rubisco (approximately 55 kDa).

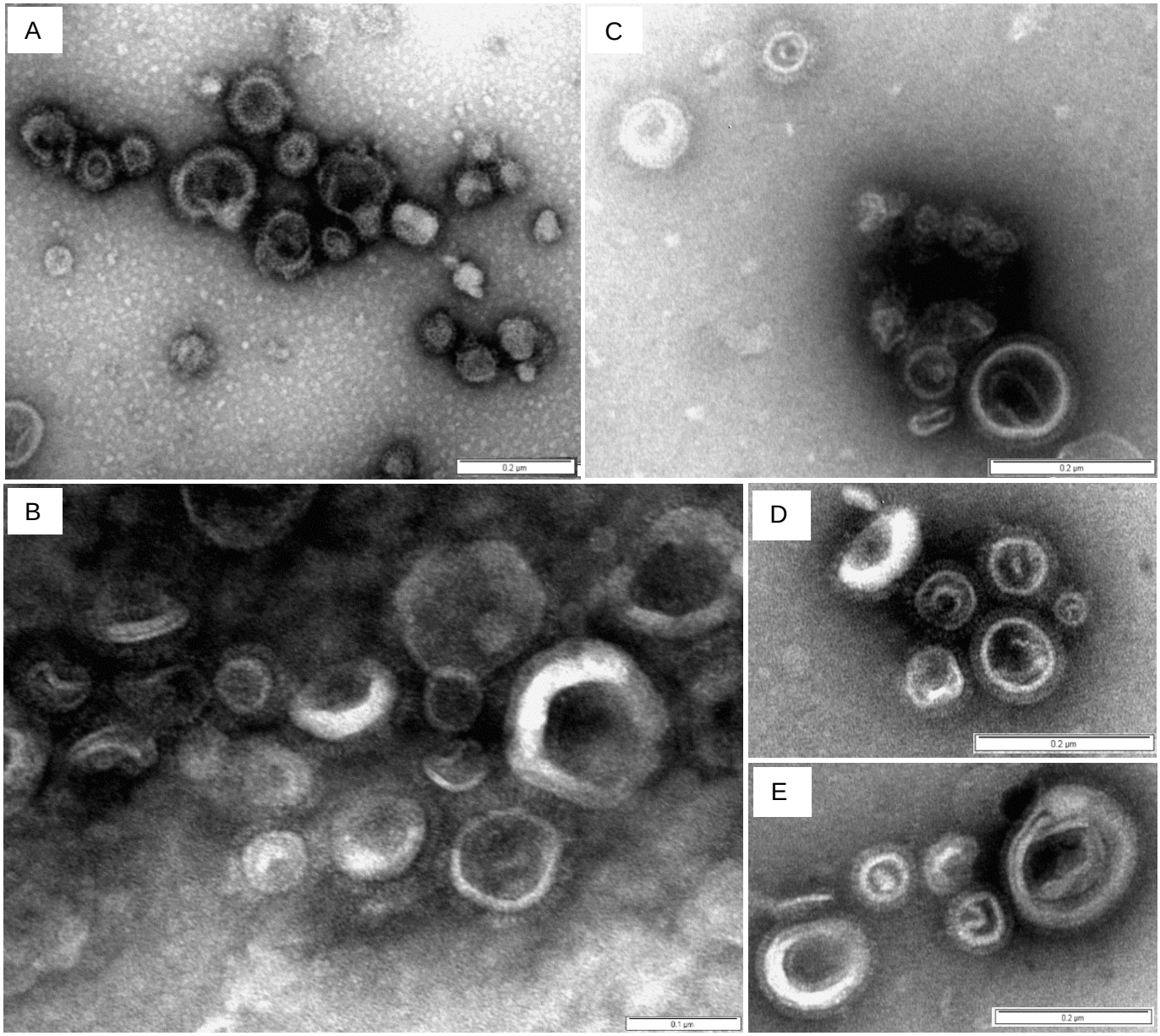


Figure 3.6: Negatively-stained transmission electron microscopy (TEM) images of plant-produced H6 avian influenza VLPs. Sub-lineage I H6 VLPs are depicted in A and B, while sub-lineage II H6 VLPs are depicted in C to E. The bar represents 0.2 µm (A, C, D, E) or 0.1 µm (B).

3.3.2.2. IMAC purification of N-His-tagged H6 VLPs

In order to analyze the binding efficiency of the His-tagged protein to the metal ions immobilized on an IMAC resin, the clarified extract (Tris buffer) was purified using pre-packed Protino® Ni-TED 2000 columns (Macherey-Nagel). Making use of immunodetection with an anti-His-HRP antibody, the N-His-tagged protein was detected in two of the elution fractions analyzed (E2 and E3), although protein recovery was poor with most of the target protein being detected in the flow through and wash fractions (Figure 3.7). A different chelator, namely IDA, was subsequently tested for increased binding efficiency by comparing Protino® Ni-TED and Ni-IDA (Macherey-Nagel) resins, using both Tris and Bicine extraction buffers for each resin. According to the manufacturer, the binding efficiency of the Ni-IDA resin is higher compared to that of Ni-TED (20 mg/grams (g) resin compared to 10 mg/g resin), although the specificity of the Ni-TED resin is higher compared to that of Ni IDA (a single protein binding compared to three). In this study, the amount of unbound protein (flow through fraction) was similar for both resins, using either Tris or Bicine extraction buffer, although protein recovery in the elution fraction(s) was slightly higher with the Ni-TED resin (Figure 3.8). Thus, Ni-TED resin appeared to be superior to Ni-IDA for the purification of plant-produced H6 VLPs.

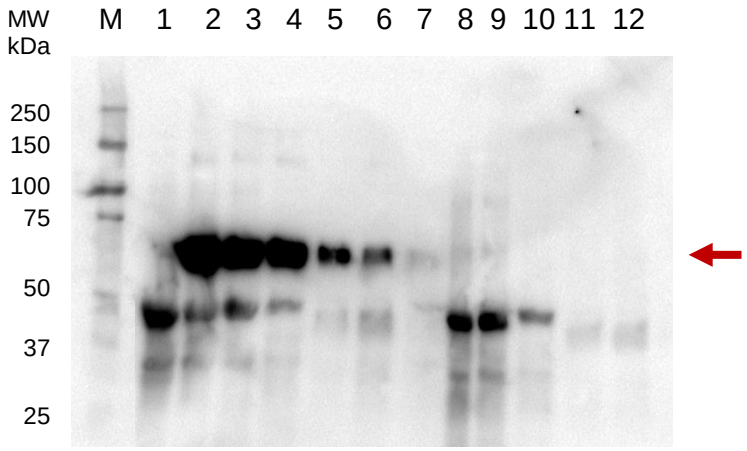


Figure 3.7. Immunodetection of sub-lineage I H6 hemagglutinin (HA_0) with a histidine (His)-tag at the N-terminus ($H6_{HA}S1C\text{-}N_{His}$) or C-terminus ($H6_{HA}S1C\text{-}C_{His}$) at different stages of immobilized metal ion affinity chromatography (IMAC) purification. Protino® Ni-TED resin was utilized for IMAC purification. Lane 1: negative control (pEAQ-HT-empty); lanes 2 to 7: $H6_{HA}S1C\text{-}N_{His}$ detected in the clarified, flow through (FT), wash (W), elution (E) 2, E3 and E4 fractions, respectively; lanes 8 to 12: $H6_{HA}S1C\text{-}C_{His}$ not detected in the clarified, FT, W, E2, E3 and E4 fractions, respectively. M: WesternC protein molecular weight marker. The arrow indicates the position of the target protein (approximately 63 kDa).

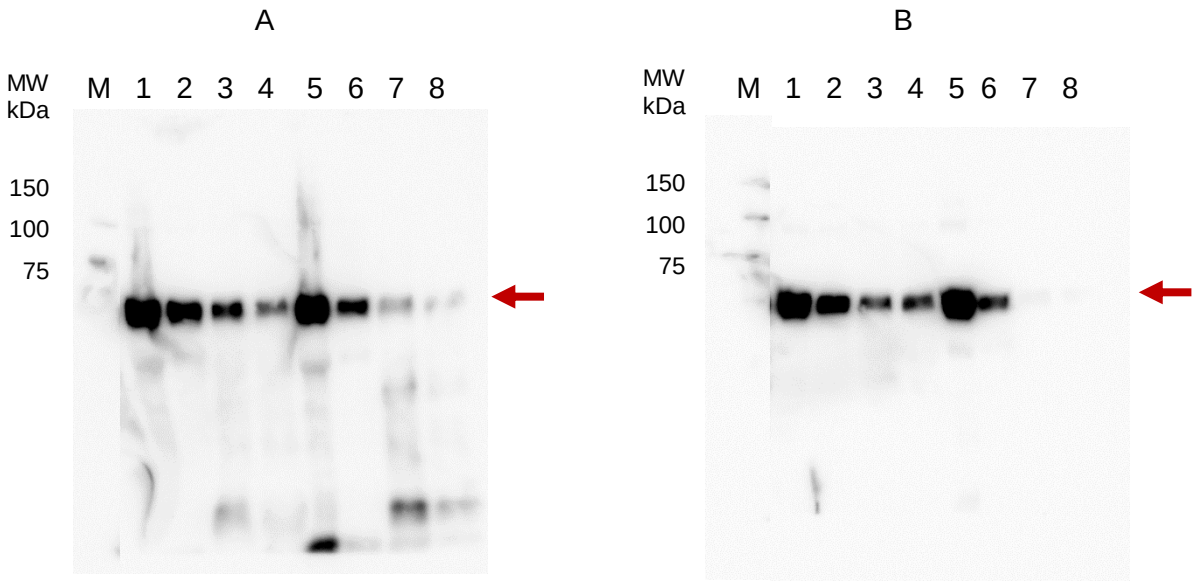


Figure 3.8. Immunodetection (composite image) of N-histidine (His)-tagged sub-lineage I H6 hemagglutinin (HA_0) ($H6_{HA}S1C\text{-}N_{His}$) purified via immobilized metal ion affinity chromatography (IMAC) using different resins. Proteins were extracted with Tris (A) or Bicine buffer (B) and Protino® Ni-TED or Ni-IDA resin were utilized for IMAC purification. Lanes 1 to 4: Ni-TED, $H6_{HA}S1C\text{-}N_{His}$ detected in the flow through (FT), wash (W), elution (E) 2 and E3 fractions, respectively; lanes 5 to 8: Ni-IDA, $H6_{HA}S1C\text{-}N_{His}$ detected in the FT, W, E2 and E3 fractions, respectively. M: WesternC protein molecular weight marker. The arrow indicates the position of the target protein (approximately 63 kDa).

To determine whether column overloading contributes to the presence of the target protein in the preceding fractions, a larger scale purification of histidine-tagged H6 VLPs (310 ml of clarified plant extract) was subsequently carried out on the ÄKTA™ Avant 150 (GE Healthcare Life Sciences) making use of Protino® Ni-TED resin with a 7 ml bed volume. However, similar results were obtained with most of the protein being lost in the flow through and wash steps (Figure 3.9), suggesting that column overloading is not the cause of the poor protein recovery obtained. The target protein was detected in all the elution fractions (Figure 3.9) and intact influenza VLPs were present in abundance, as confirmed by TEM analysis (data not shown). Other modifications could be introduced into the purification method (for example testing a different chelator, adjusting the buffer compositions, or elution using low pH instead of imidazole), although it was suspected that the 6xHis-tag is only partially exposed and/or that the stereochemistry of the H6 VLPs affect the interaction between the histidines and the Ni^{2+} metal ions, leading to inefficient column binding and poor recovery of the target protein.

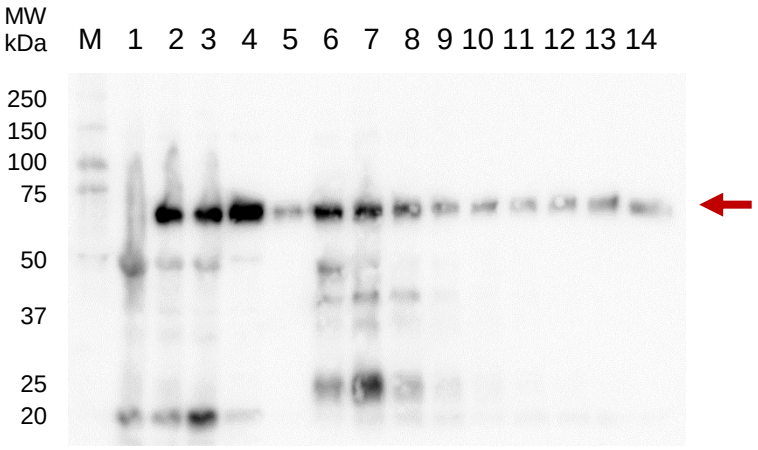


Figure 3.9: Immunodetection of ÄKTA-purified N-histidine (His)-tagged sub-lineage I H6 hemagglutinin (HA_0) ($H6_{HA}S1C\text{-}N_{His}$). Lane 1: negative control (pEAQ-HT-empty); lane 2: crude protein; lane 3: unbound fraction (flow through); lane 4: wash fraction; lanes 5 to 14: elution fractions 1 to 10. M: WesternC protein molecular weight marker. The arrow indicates the position of the target protein (approximately 63 kDa).

In an attempt to improve the accessibility of the affinity tag for more efficient column binding, a linker sequence encoding four Glycine and one Serine residues, was subsequently added between the N-terminal histidine sequence and the sub-lineage I H6 HA_0 gene sequence ($H6_{HA}S1C\text{-}N_{His}$-L) using PCR. The addition of the linker increased expression levels moderately in comparison to the untagged and N-His-tagged constructs (Figure 3.10A), although purification with IMAC was still not efficient (Figure 3.10B). The I-TASSER (Iterative Threading ASSEmbly Refinement) server, an online resource for the prediction of protein structure and function, was subsequently utilized to investigate the exposure of the His-tag on the N-His-tagged sub-lineage I insert and the N-His-tagged sub-lineage I insert containing a linker (Zhang *et al.*, 2017; Yang and Zhang, 2015). Based on the predicted solvent accessibility score, which ranges from 0 (buried residue) to 9 (highly exposed residue), two of the six His residues of the N-His tagged sub-lineage I protein ($H6_{HA}S1C\text{-}N_{His}$) are buried (score < 2 according to I-TASSER) and 4/6 His residues received a predicted solvent associability score of four or less (Figure 3.11 A). With the addition of the GGGGS linker ($H6_{HA}S1C\text{-}N_{His}$-L) the number of predicted buried residues reduced to one, although the 5/6 His residues received a predicted solvent associability score of four or less (Figure 3.11 B). For the sub-lineage II N-His-tagged protein, two of the six His residues were buried and 4/6 His residues received a predicted solvent associability score of four or less (Figure 3.11) Thus, it is likely that insufficient exposure of the 6XHis-tag contributes to the poor recovery of the target protein obtained in this study.

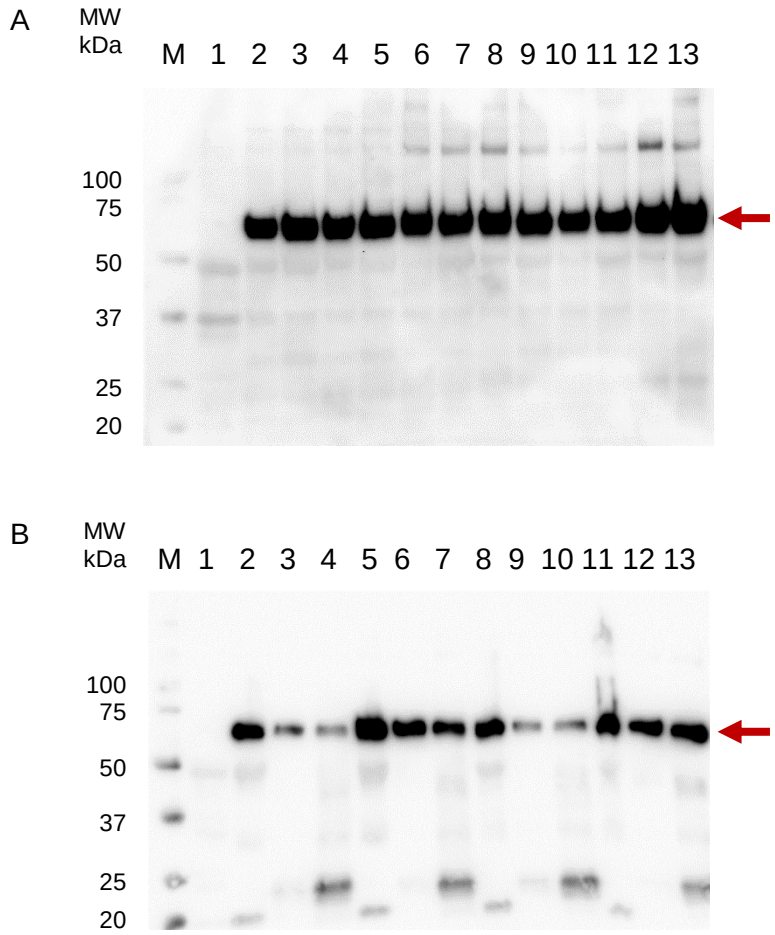


Figure 3.10. Immunodetection images demonstrating the difference in yield obtained with the addition of a N-terminal histidine (His)-tag ($H6_{HA}S1C\text{-}N_{His}$) and incorporation of a linker sequence between the N-His-tag and the sub-lineage I H6 hemagglutinin (HA_0) gene ($H6_{HA}S1C\text{-}N_{His}\text{-}L$). Proteins were extracted with Tris or PBS and purified using differential centrifugation (A) or immobilized metal ion affinity chromatography (IMAC) (Protino® Ni-TED) (B). A) Lane 1: negative control (pEAQ-HT-empty); lanes 2 & 3: $H6_{HA}S1C$, Tris, iodixanol fractions 10 & 11; lanes 4 & 5: $H6_{HA}S1C$, PBS, iodixanol fractions 10 & 11; lanes 6 & 7; $H6_{HA}S1C\text{-}N_{His}$, Tris, iodixanol fractions 10 & 11; lanes 8 & 9: $H6_{HA}S1C\text{-}N_{His}$, PBS, iodixanol fractions 10 & 11; lanes 10 & 11: $H6_{HA}S1C\text{-}N_{His}\text{-}L$, Tris, iodixanol fractions 10 & 11; lanes 12 & 13: $H6_{HA}S1C\text{-}N_{His}\text{-}L$, PBS, iodixanol fractions 10 & 11. B) Lane 1: pEAQ-HT; lanes 2 to 4: $H6_{HA}S1C\text{-}N_{His}$, Tris, flow through (FT), elution 2 (E2) and elution 3 (E3) fractions; lanes 5 to 7: $H6_{HA}S1C\text{-}N_{His}$, PBS, FT, E2 and E3; lanes 8 to 10: $H6_{HA}S1C\text{-}N_{His}\text{-}L$, Tris, FT, E2 and E3; lanes 11 to 13: $H6_{HA}S1C\text{-}N_{His}\text{-}L$, Tris, FT, E2 and E3. M: WesternC protein molecular weight marker. The arrow indicates the position of the target protein (approximately 63 kDa). Tris: Tris(hydroxymethyl)aminomethane; PBS: Phosphate buffered saline.

A

Predicted Solvent Accessibility

```
                    20                  40                  60
                    |                   |                   |
Sequence    HHHHHHDKICIGYHANNSTTQVDTILEKNITVTHSIELLETQKEERFCRVLNKAPLDLRECTIEGWMLGNP
Prediction  842461310000010143343021024670413202412345333300416231102035010000000 10
            Values range from 0 (buried residue) to 9 (highly exposed residue)
```

B

Predicted Solvent Accessibility

```
                    20                  40                  60
                    |                   |                   |
Sequence    HHHHHHGGGGSDKICIGYHANNSTTQVDTILEKNITVTHSIELLETQKEERFCRVLNKAPLDLRECTIEGW
Prediction  744243334402100000101433430210246704132024123453333004162311020350100 00
            Values range from 0 (buried residue) to 9 (highly exposed residue)
```

C

Predicted Solvent Accessibility

```
                    20                  40                  60
                    |                   |                   |
Sequence    HHHHHHDKICIGYHANNSTTQVDTILEKNVTVTHSIELLETQKEERFCKILNKAPLDLGECTIEGWILGNP
Prediction  842461310000010143343021024670413202412345333300416231103035010000000 10
            Values range from 0 (buried residue) to 9 (highly exposed residue)
```

Figure 3.11. Images of the predicted solvent accessibility scores according to I-TASSER for selected N-histidine (His)-tagged H6 hemagglutinin (HA_0) proteins. The N-terminal amino acids of N-His-tagged sub-lineage I H6 HA_0 ($H6_{HA}S1C\text{-}N_{His}$) (A), N-His-tagged sub-lineage I H6 HA_0 with a linker ($H6_{HA}S1C\text{-}N_{His}\text{-}L$) (B) and N-His-tagged sub-lineage II H6 HA_0 ($H6_{HA}S2C\text{-}N_{His}$) (C) are depicted here.

3.3.2.3. Tangential flow filtration (TFF) purification of N-His-tagged sub-lineage I H6 VLPs

Following the poor protein recovery obtained using IMAC, an alternative commercially scalable membrane-based purification method was explored, namely depth filtration followed by tangential flow filtration (TFF). In this study, vacuum infiltration clarified plant extract was subjected to depth filtration (Sartoclean GF Sterile MidiCap, Sarotrius Stedim Biotech), followed by TFF using the Minimate™ TFF capsule with Omega 300K membrane (PALL Life Sciences, PALL South Africa (Pty) Ltd., Midrand, South Africa). The Pierce™ Micro BCA protein assay kit was used to determine the total soluble protein in the TFF purified sample, the percentage that H6 HA_0 comprises of the total soluble protein by quantification of iodixanol purified (fraction 10) TFF dilutions (1 in 2, 1 in 4 and 1 in 10) and the protein yield per kg biomass. In this experiment, using vacuum infiltration (OD_{600} = 0.4; 30 mbar, 4 minute

holding time) and depth filtration/TFF, N-His-tagged H6 HA_0 is conservatively estimated to account for 1.4% to 2.5% of the total soluble protein in the TFF purified sample (2.1 mg), with the protein yield conservatively estimated at 158.81 mg/kg leaf material. In a previous study where pEAQ-HT, *A. tumefaciens* strain AGL-1, vacuum infiltration and Tris extraction buffer was employed in the transient production of hemagglutinin-based influenza VLPs in *N. benthamiana* (but without the addition of a His-tag and co-expression with influenza M2 as conducted in this study), the estimated protein yield of H5 HA was estimated at 50 mg/kg fresh weight leaf material (D'Aoust *et al.,* 2008). In another study involving pEAQ-HT to express bluetongue VLPs in *N. benthamiana*, an estimated total protein yield of over 200 mg/kg was reported for all four of the particulates (Thuenemann *et al.,* 2013). Thus, the protein yield of H6 HA_0 obtained in this study using vacuum infiltration and TFF, which are commercially scalable methods, was comparable to previous experimental studies involving the pEAQ-HT plant expression vector.

3.4. Conclusions

DSP includes protein extraction and purification and accounts for a significant proportion of manufacturing costs for plant-based expression systems and keeping the processing steps to a minimum is critical in ensuring a cost-effective product. The extraction buffer provides a stable pH environment and salinity required for protein stability and, therefore, plays an important role in heterologous protein expression. To determine the effect of extraction buffers on protein yield, three buffers that have previously been reported for the extraction of VLPs, namely Tris (pH 8.0), Bicine (pH 8.4) and PBS (pH 7.4) (Landry *et al.,* 2010; Thuenemann *et al.,* 2013; Ruiz *et al.,* 2018), were tested for efficiency in this study. It was determined that the Tris buffer was considered to be more suited for the extraction of plant-produced H6 influenza VLPs in comparison to the Bicine buffer, as it yielded the most abundant functional H6 VLPs. In addition, PBS buffer was found to be at least as effective as Tris buffer (if not slightly superior) for the extraction of H6 influenza VLPs. PBS is widely used as a placebo in clinical and animal trials and extraction using this buffer could exclude the necessity for buffer exchange by dialysis in PBS prior to administration of the antigen. Therefore, the use of PBS as extraction buffer is beneficial for the yield of H6 influenza VLPs and could reduce the number of processing steps, which is advantages in terms of process time and production costs.

IMAC purification is a simple and scalable method that could potentially reduce the number of purification steps, ensuring a cost-effective manufacturing platform. As such, the effect of the addition of a poly-His tag on expression levels and VLPs assembly was investigated in this study. The incorporation of a poly-His-tag at the C-terminus of the sub-lineage I chicken codon optimized synthetic gene failed to yield the target protein, while an N-terminus His-tag increased protein expression and VLP assembly moderately for the sub-lineage I chicken codon optimized gene and more drastically for sub-lineage II chicken codon optimized gene. Abundance of sub-lineage II VLPs, as determined by TEM, was only attained with the addition of the N-terminal His-tag. The plant-produced N-His-tagged sub-lineage I and II influenza VLPs agglutinated chicken erythrocytes, with hemagglutination titers of 10 $\log_2$ and 8 $\log_2$, respectively, thereby confirming that the VLPs are functional and that the addition of the His-tag did not alter its conformation. The addition of the Kozak consensus sequence, which is frequently added immediately upstream of the start codon to enhance translation efficiency, did, however, result in a slight decline in expression of the target protein and a reduction in hemagglutination titer of 1 $\log_2$ was observed for both sub-lineage I and II N-His-tagged VLPs. Thus, for the expression of H6 influenza VLPs, the Kozak sequence used in this study has a negative effect on expression levels, while the addition of a N-His-tag is advantages for protein expression without negatively affecting VLP assembly and function.

IMAC purification of the His-tagged proteins were subsequently investigated by making use of Protino® Ni-TED or Ni-IDA resin. Protein recovery was improved using the Protino® Ni-TED resin, although most of the target protein was lost in the flow through and wash fractions, using either Tris or PBS extraction buffers. In an effort to improve the efficiency of IMAC purification, a linker sequence was added between the N-His-tag and the sub-lineage I H6 HA_0 gene to improve the flexibility and/or exposure of the His-tag for more effective binding to the metal ions. The addition of a linker sequence increased expression levels moderately in comparison to the N-His-tagged sub-lineage I construct, but did not improve the efficiency of IMAC purification using Ni-TED resin. The protein sequences of N-His sub-lineage I and N-His-linker sub-lineage I was submitted to I-TASSER, an online resource for the prediction of protein structure and function. The predicted solvent accessibility scores, with 0 indicating buried residues and 9 indicating highly exposed residues, for the six His residues of the N-His-tagged sub-lineage I protein are 8, 4, 2, 4, 6 and 1, and was only slightly improved with the addition of the linker sequence. Thus, in this study, the poor recovery of N-his-tagged proteins are most probably due to the tag not being sufficiently exposed to bind effectively to the Ni-TED resin. Nevertheless, this study indicates the potential of using IMAC for the purification of plant-produced H6 influenza VLPs. In future, improving the exposure of the

N-terminal His-tag, for example by extending the linker and/or poly-histidine sequences, by investigating alternative linker sequences and/or by employing protein modelling to determine the location best suited for incorporation of a His-tag, could result in efficient protein recovery and make IMAC a viable simple and cost-effective purification method for plant-produced H6 influenza VLPs.

To test plant-based expression of H6 VLPs on a larger scale, vacuum infiltration was used in combination with depth filtration/ TFF. Conservatively estimated, the target protein (N-His-tagged sub-lineage I) accounted for 1.4% to 2.5% of the total soluble protein, with a protein yield estimated at 158.81 mg/kg fresh weight leaf material. The yield obtained in this study is comparable to previous reports of 50 mg/kg and over 200 mg/kg for transient VLP production in *N. benthamiana* using the pEAQ-HT expression vector, indicating the efficiency of H6 VLP production using commercially scalable infiltration and purification methods.

Chapter 4
Efficacy testing of plant-produced H6 sub-lineage I and II avian influenza VLPs in chickens

4.1. Introduction

The H6 subtype avian influenza is one of the most prevalent subtypes in poultry and is endemic in many regions, including South Africa (Chin *et al.,* 2002; Liu *et al.,* 2003; Woolcock *et al.,* 2003; Abolnik, 2007a; Abolnik, 2007b; Alexander, 2007; Brown, 2010; Wang *et al.,* 2014; Wang *et al.,* 2016). LPAI infection typically results in increased mortalities, reduced egg production and mild to severe respiratory disease accompanied by increased secondary bacterial infections that require antibiotic treatment, although some infected poultry might be asymptomatic (Kinde *et al.,* 2003; Woolcock *et al.,* 2003; Kishida *et al.,* 2004; Suarez, 2017). In addition to the significant risk of LPAI viruses contributing to the genetic diversity of influenza subtypes with pathogenic potential due to reassortment, several avian LPAI viruses (including H6N1, H7N9, H9N2 and H10N8) have infected humans, resulting in mild to severe forms of disease (Guan *et al.,* 1999; Hoffmann *et al.,* 2000; Gao *et al.,* 2013; Wei *et al.,* 2013; Chen *et al.,* 2014). Of the LPAI subtypes, H6 in particular is considered to pose a potential threat to human health (Munster *et al.,* 2007; Gillim-Ross *et al.,* 2008; Nam *et al.,* 2011; Zhang *et al.,* 2011; Wang *et al.,* 2014; Ni *et al.,* 2015; Wang *et al.,* 2015; Zou *et al.,* 2016; Ge *et al.,* 2017). Due to the potential economic and social impact of LPAI, the control and surveillance of LPAI viruses are of great importance.

Vaccination against avian influenza can be a very effective tool when used as part of a comprehensive control program and can be applied preventatively, routinely, or in cases of emergency (Swayne *et al.,* 2011; Swayne and Kapczynski, 2017). The vast majority of registered AI vaccines used in the field, typically in the commercial poultry sector, are whole inactivated virus vaccines produced in specific-pathogen-free (SPF) chicken eggs and formulated with oil-emulsion adjuvants to enhance its immunogenicity (Swayne and Kapczynski, 2017). The goal of avian influenza vaccination is to elicit an immune response that confers protection against disease (morbidity and mortality) and ideally, the prevention of infection. Protection against clinical disease and field viral shedding is primarily attained through systemic humoral immunity, although cell-mediated immunity also reportedly plays a role (Swayne *et al.,* 2011; Swayne and Kapczynski, 2017). The major surface glycoprotein hemagglutinin (HA) is the primary target for neutralizing antibodies and the HA gene has a characteristically high mutation rate to escape the host's immune response, which is exacerbated under vaccination pressure. Antigenic differences between the HA of vaccine

and seed strains can significantly reduce the effectiveness of the vaccine and it is, therefore, recommended that vaccine strains should be re-evaluated periodically for efficacy against circulating viruses and updated as needed (Swayne *et al.,* 2006; Swayne and Kapczynski, 2017; The World Organisation for Animal Health (OIE), 2018).

Under experimental conditions, the prevention of mortality and morbidity and the prevention or reduction in viral replication and shedding from the respiratory and gastrointestinal tracts, are protective criteria that evaluates the capacity of the avian influenza vaccine to limit virus spread (Swayne and Kapczynski, 2017). For LPAI viruses, clinical signs are not typically produced under experimental conditions, and the main determinant of vaccine efficacy is a reduction in viral shedding titer, as well as a shortened duration of viral shedding, in comparison to a non-vaccinated control group (Swayne and Kapczynski, 2017; OIE, 2018). A reduction in replication and shedding titers from the respiratory and gastrointestinal tracts should be at least 100-fold less virus in comparison to the non-vaccinated control group, or statistical significant differences between the vaccinated and non-vaccinated groups should be demonstrated for a vaccine to be deemed effective (Swayne and Kapczynski, 2017). Such a reduction in viral shedding is best attained when the HA of the vaccine and challenge virus are genetically or antigenically closely related, which again highlights the importance of periodic updating of avian influenza vaccines to limit field virus spread (Swayne and Kapczynski, 2017).

In South Africa, the chicken industry has been beset by sporadic outbreaks of LPAI H6N2 since the early 2000s and two distinct lineages (H6N2 sub-lineage I and II) were identified from the onset (Abolnik *et al.,* 2007a; Abolnik *et al.,* 2007b). At the time, an autogenous inactivated oil-emulsion egg-based whole inactivated virus vaccine derived from a H6N2 sub-lineage I field strain isolated in 2002 (AVIVAC® AI; Deltamune (Pty) Ltd., South Africa) was commercialized to protect flocks. This commercial vaccine is still in use at the time of writing, albeit under strictly regulated conditions. However, after more than a decade of vaccination, extensive evolutionary changes have been documented for H6N2 field viruses, especially in sub-lineage I isolates, and mutations that have previously been associated with human receptor-binding and/or virulence in mammalian species have been identified (Rauff *et al.,* 2016; Abolnik *et al.,* 2019). Prior to the present study, the efficacy of the commercial H6N2 vaccine against circulating field strains had not been determined in a clinical study, nor have any subsequent field isolates been developed as a replacement vaccine seed strain.

One of the alternatives to traditional egg-based influenza vaccine production that has become increasingly popular in recent years is plant-based virus-like particle (VLP) vaccines. VLP-based vaccines offer superior immunogenicity (both cellular and humoral immune responses) and antigen stability compared to other subunit vaccines, are considered to be safer in comparison to attenuated or inactivated vaccines, and the absence of core genetic material enables differentiation between infected and vaccinated animals (DIVA) in the field (Noad and Roy, 2003; Bright *et al.,* 2007; Quan *et al.,* 2007; Chen and Lai, 2013; Liu *et al.,* 2013). The transient production of VLPs in plants offer additional advantages of cost-effective scalability, glycosylation of the target protein, which is required for immunogenicity and stability of VLP antigens, and unprecedented speed of production (D'Aoust *et al.*, 2010; Kim *et al.,* 2014; Kolotilin *et al.,* 2014; Walwyn *et al.,* 2015; Nandi *et al.,* 2016). Medicago's seasonal and pandemic human hemagglutinin (HA)-based VLP influenza vaccines transiently produced in plants have shown good safety and immunogenicity in pre-clinical and clinical tests (Phase I and II) and an estimated 30 million doses of vaccine could be produced within 3 months once the HA sequence of the influenza virus is available (D'Aoust *et al.,* 2008; Landry *et al.,* 2010; Margolin *et al*., 2018). The short production time frame of plant-based expression is of great importance in the case of an influenza pandemic and facilitates prompt updating of influenza vaccines to match AI strains currently circulating in a specific country.

This chapter describes the efficacy testing of the sub-lineage I and II H6 VLPs transiently produced in *Nicotiana benthamiana* (Chapters 2 and 3). As the sub-lineage II construct containing a N-Histidine (His)-tag was not available for use at the time of the animal trial, the untagged sub-lineage I and II VLPs, respectively, were tested for efficacy. Each of the VLP types were formulated with an oil-emulsion adjuvant (ISA 71 VG; Seppic, France) and tested for their capacity to effectively reduce viral shedding in prime-boost vaccinated specific-pathogen-free (SPF) chickens upon challenge with a heterologous 2016 field virus. Results were compared to the H6N2 commercial whole inactivated virus vaccine and a non-vaccinated control group. The primary objective was to determine the quantity of virus shed from the respiratory and gastrointestinal tracts, respectively, up to 21 days post viral challenge, using a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay targeting the influenza Matrix gene. In addition, humoral immune responses were evaluated after the first vaccination (pre-booster), after the second vaccination (pre-challenge) and two weeks after viral challenge. HA-specific antibody responses were determined using hemagglutination inhibition (HI) assays, while nucleoprotein (NP)-specific antibody responses were determined using commercial IDEXX Influenza A Antibody test kits.

The results of the H6 sub-lineage I VLP vaccine (in comparison to the commercial vaccine and non-vaccinated control groups) has been published (Smith *et al.,* 2019) and is referred to during this chapter, particularly relating to the details of the methodology.

4.2. Materials and methods

4.2.1. Ethical considerations

All procedures were pre-approved by the Department of Agriculture, Forestry and Fisheries (DAFF) (Reference number: 12/11/1/1/8), the Animal Ethics Committee (AEC) of UP (Reference number: V075-17), and the CSIR Research Ethics Committee (Reference number: 230/2017) (Appendix A).

4.2.2. The production of H6 sub-lineage I and II VLPs in *N. benthamiana*

The plant-produced influenza H6 sub-lineage I and II VLPs were manufactured at the Council for Scientific and Industrial Research (CSIR), Biosciences, Pretoria, South Africa. The H6 VLPs are based on the HA gene of the most recent sub-lineage I (A/chicken/South Africa/N2826/2016 (H6N2)) and sub-lineage II (A/Chicken/South Africa/BKR4/2012 (H6N2)) H6N2 viruses that were available at the time of gene design (Chapters 2 and 3). To enhance expression levels, the sub-lineage I ($H6_{HA}S1C$) and II ($H6_{HA}S2C$) H6 HA_0 protein were each co-expressed with influenza M2 from A/New Caledonia/20/1999 (H1N1) (Genbank accession number HQ008884) (Jutras *et al.,* 2015; Chapter 2). The respective H6 HA_0 and M2 genes, which were codon optimized and synthesized by Bio Basic Inc. (Toronto, Ontario, Canada), contained the *Mus musculus* monoclonal antibody heavy chain variable region signal peptide (O'Hara *et al.,* 2012) and *Age*I and *Xho*I restriction enzyme recognition sites at the 5'- and 3'-terminals, respectively, for cloning into pEAQ-HT (Sainsbury and Lomonossoff, 2008), as described previously (Chapter 2, section 2.2.2).

Validated *Agrobacterium tumefaciens* (AGL-1) transformants carrying the expression vectors pEAQ-HT+M2 (plant codon optimized influenza M2), pEAQ-HT+$H6_{HA}S1C$ (sub-lineage I chicken codon optimized H6 HA_0 gene), and pEAQ-HT+$H6_{HA}S2C$ (sub-lineage II chicken codon optimized H6 HA_0 gene), respectively, were prepared for agroinfiltration, as previously described (Chapter 2, section 2.2.4). Briefly, selected AGL-1 verified clones were subcultured, grown overnight at 28°C in Lysogeny broth (LB) containing 30 µg/ml rifampicin and 50 µg/ml kanamycin, harvested by centrifugation (8,000 x g, 8 minutes), and suspended in infiltration buffer (10 mM 2-N-morpholino-ethanesulfonic acid (MES), 20 mM magnesium sulfate ($MgSO_4$), pH 5.6) supplemented with 200 µM acetosyringone (Smith *et al.,* 2019). Each of the *A. tumefaciens* suspensions were diluted to obtain a final optical density (OD_{600})

of 1.5, whereafter both the pEAQ-HT+H6$_{HA}$S1C and pEAQ-HT+H6$_{HA}$S2C suspensions were mixed in a 1:1 ratio with the pEAQ-HT+M2 suspension and incubated at room temperature for at least one hour. The leaves of five-to-eight-week glycol-engineered *N. benthamiana* plants (Strasser *et al.,* 2008) were infiltrated with the respective *A. tumefaciens* suspensions (namely 1. pEAQ-HT+H6$_{HA}$S1C with pEAQ-HT+M2, and 2. pEAQ-HT+H6$_{HA}$S2C with pEAQ-HT+M2) using a syringe without a needle.

Six days after infiltration, approximately 40 grams of infiltrated leaves were harvested for sub-lineage I and II H6 HA_0, respectively, and homogenized in two volumes of Tris buffer (Tris(hydroxymethyl)aminomethane); Landry *et al.,* 2010) containing proteinase inhibitor cocktail (P2714, Sigma-Aldrich, St. Louis, Missouri, United States of America) using a Matstone DO9001 Juicer, as previously described (Chapter 2 section 2.2.4; Smith *et al.,* 2019). Briefly, the homogenate was clarified through a double layer of cheese cloth and loaded onto a 20 to 60% Iodixanol (OptiPrep™, Sigma Aldrich) density gradient for purification. Following ultracentrifugation (32,000 x g, 2 hours, 10^{o}C, Beckman Coulter Ultra-centrifuge Optima L90K), fractions were collected from the bottom of the Thinwall Ultra-Clear™ tube (Beckman Coulter) and the three fractions containing the most abundant H6 protein were pooled. SnakeSkin Dialysis Tubing (10K MWCO, 35 mm dry I.D.; Thermo Fisher Scientific™, Waltham, Massachusetts, United States of America) was used to dialyse the partially purified H6 VLPs overnight in phosphate buffered saline (PBS, pH 7.4), whereafter Trehalose dihydrate (15% w/v) (Sigma-Aldrich) was added as stabilizing agent (Lynch *et al.,* 2012). The products were stored at 4^{o}C until use. To confirm the expression of H6 HA_0 and the formation of intact functional VLPs, SDS-PAGE analysis, immunoblotting, transmission electron microscopy (TEM), hemagglutination, and hemagglutination inhibition (HI) assays were performed as described previously (Chapter 2, Section 2.2.5).

4.2.3. Efficacy study in SPF chickens

4.2.3.1. Experimental Animals

The Veterinary Faculty's Poultry Biosafety Level 3 facility, University of Pretoria (UP), was used for the vaccine-challenge study in chickens (*Gallus gallus*). At six weeks of age, certified SPF White Leghorn type chickens (n = 48) purchased from Avi-Farms (Pty) Ltd. (Pretoria, South Africa) were numbered individually and randomly assigned to four enclosed pens, each containing 12 chickens (treatment groups A, B, C and D). After viral challenge, the chickens were assigned to eight designated isolators (treatment group A, B, C or D), each housing 6 chickens. Layer grower feed (Nova Feeds, Pretoria, South Africa) and water was provided *ad libitum* for the duration of the trial, with daily checks carried out.

4.2.3.2. VLP vaccine formulation

Partially-purified H6 sub-lineage I and II VLPs were tested by hemagglutination and HI assays and stored at 4°C until use. For sub-lineage I VLPs, the vaccine dose of 35.7 µl of plant leaf extract was calculated to corresponded to an HI titer of 1:1024 (10 $\log_2$) or 768 HAU/300 µl, while the vaccine dose of 150 µl of sub-lineage II VLPs was calculated to correspond to an HI titer of 1:16 (4 $\log_2$) or 48 HAU/300 µl. On the day of vaccination, the partially-purified plant-produced H6 sub-lineage I VLPs were diluted in PBS and mixed in a 1:1 ratio with Montanide™ ISA 71 VG adjuvant (Seppic, France), whereas the H6 sub-lineage II VLPs were mixed in a 1:1 ratio with the Montanide™ ISA 71 VG adjuvant without dilution in PBS to obtain the maximum possible antigenic mass dose.

The commercial H6N2 oil-emulsion inactivated vaccine (AVIVAC® AI) (batch No. 60076, expiration date 05/2019) was purchased under a DAFF Section 20 permit from the manufacturer. The vaccine seed strain is A/Chicken/South Africa/W-04/2002 (H6N2), a sub-lineage I virus (Figure 4.1) (Rauff *et al.,* 2016). This egg-based inactivated vaccine is adjuvanted with oil and consists of 20% antigen, 72% white oil [v/v], 6% Arlacel [v/v], and 2% Tween 80 [v/v] (Rauff, 2015). According to the label, the mean embryo infectious dose (EID_{50}) of the commercial vaccine is ≥ 10^8 per recommended dose (0.5 ml) and results in a high immune response (HI titer ≥ 6 $\log_2$).

4.2.3.3. Challenge virus

In this study, the field strain used in the design of the H6 sub-lineage I VLP vaccine (A/chicken/South Africa/N2826/2016 (H6N2)), could not be used for challenge as the isolate, cultured at UP from a flock infected with multiple pathogens, was contaminated with a virulent Newcastle disease virus. Instead, another sub-lineage I field virus (A/chicken/South Africa/H44954/2016 (H6N2) (Table 4.1; Figure 4.1) was obtained from RCL Foods (Pty) Ltd. (South Africa). As described in Smith *et al.* (2019), this virus was isolated from tracheal samples of 56-week-old commercial layer hens in Pietermaritzburg, KwaZulu-Natal Province, in November 2016 and, despite the flock being vaccinated with the commercial H6N2 vaccine (AVIVAC® AI), showed a 10% drop in egg production. On post-mortem, mild tracheitis and secondary *Escherichia coli (E. coli)* peritonitis and airsacculitis were observed. In the HA protein, the challenge strain (A/chicken/South Africa/H44954/2016 (H6N2)) shares 95.8% amino acid sequence identity with the sub-lineage I VLP strain (A/chicken/South Africa/N2826/2016), 85.7% amino acid sequence identity with the sub-lineage II VLP strain (A/chicken/South Africa/N2826/2016), and 91.5% amino acid sequence identity with the commercial vaccine strain (A/chicken/South Africa/W-04/2002) (Table 4.1). The challenge

virus was propagated further at UP in SPF embryonated chicken eggs and the method of Reed and Muench (1938) was used to determine the EID_{50}. Stock with a titer of $10^{6.8}$ EID_{50} was aliquoted and frozen at -80°C until use. On the day of challenge, stock was thawed and diluted in OculoNasal diluent (Intervet) to a titer of 10^6 EID_{50}/0.06 ml, which corresponds to one drop in each eye. The prepared challenge material was kept on ice until administered.

Table 4.1. Pairwise amino acid distances of the hemagglutinin proteins of H6N2 strains used in this efficacy study.

Strain	**BKR4**	**N2826**	**H44954**
A/chicken/South Africa/W-04/2002 *Commercial H6N2 vaccine seed strain (sub-lineage I)*	92.9%	91.2%	91.5%
A/chicken/South Africa/BKR4/2012 *Plant-produced H6 sub-lineage II VLP vaccine*		86.2%	85.7%
A/chicken/South Africa/N2826/2016 *Plant-produced H6 sub-lineage I VLP vaccine*			95.8%

W04 is the commercial H6N2 vaccine seed strain, BKR4 was used in the design of the plant-produced sub-lineage II VLP vaccine, N2826 was used in the design of the plant-produced sub-lineage I VLP vaccine and H44954 was the challenge virus.

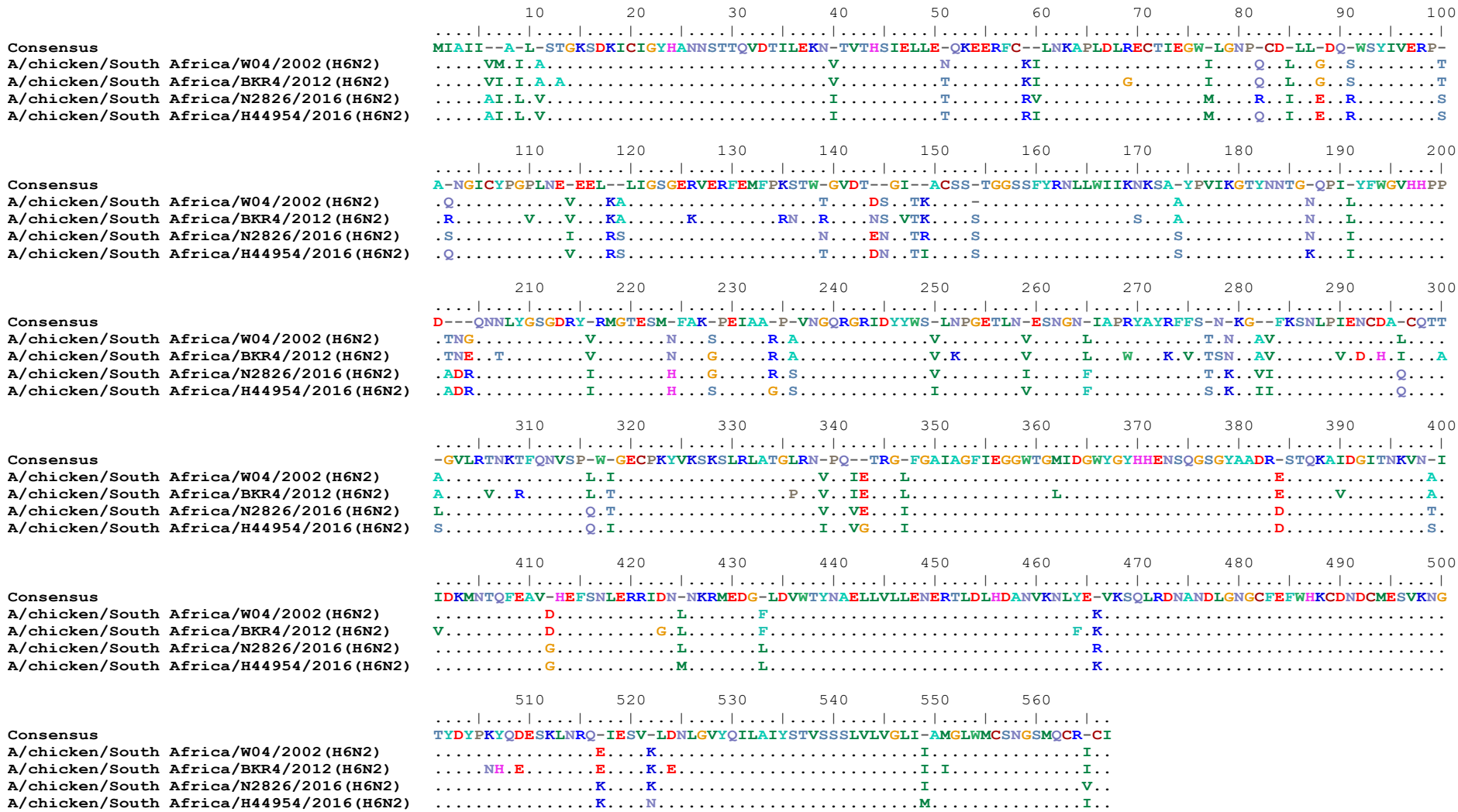


Figure 4.1. Multiple sequence alignment of the hemagglutinin proteins of the strains used in this efficacy study. W04 is the commercial H6N2 vaccine seed strain, BKR4 and N2826 were used in the design of the plant-produced sub-lineage II and sub-lineage I VLP vaccines, respectively, and H44954 was the challenge virus. Sequences are aligned to a consensus with identical residues plotted with a dot.

4.2.3.4. Experimental design

At day 0 of the study, ten chickens were randomly-selected and blood (1 ml) was sampled from the wing vein of each to confirm that the SPF chickens had no prior exposure to influenza A virus (Figure 4.2). These 10 chickens were subsequently assigned to treatment group D (non-vaccinated control). Thereafter, group A (n=12) was vaccinated intra-muscularly in the breast with 0.3 ml of the H6 sub-lineage I VLP vaccine using sterile 11-gauge needles, group B (n=12) was vaccinated intra-muscularly in the breast with 0.3 ml of the H6 sub-lineage II VLP vaccine, while group C (n=12) was vaccinated intra-muscularly in the breast with 0.5 ml of the commercial inactivated H6N2 vaccine (AVIVAC® AI). Four weeks after primary immunization, all chickens in groups A, B and C were bled as above to determine the HA- and nucleoprotein (NP)-specific antibody responses, and subsequently received a booster of the respective vaccine (Figure 4.2). Two weeks after the booster immunization, all vaccinated chickens (groups A, B and C) were bled and all birds (groups A, B, C and D) were subsequently challenged with 10^6 EID_{50} of the challenge virus via the oculo-nasal route (Figure 4.2). Chickens were observed daily throughout the trial for adverse vaccine effects and after challenge for clinical signs of disease (e.g. conjunctivitis, ocular or nasal discharge, respiratory distress such as difficulty breathing, coughing or snicking, loss of appetite, huddling, ruffled feathers or general depression). At 2, 3, 4, 7, 14 and 21 days post viral challenge (dpc), sterile plastic applicator rayon-tipped swabs (Copan Diagnostics Inc., Murrieta, California, United States of America) were used to swab the choanal cleft and cloaca of each chicken. The swabs were placed individually into 1 ml of viral transport medium (VTM), consisting of brain-heart broth, 0.1 milligrams (mg)/ml doxycycline, 0.1 mg/ml enrofloxacin, 1 mg/ml penicillin-streptomycin and 10% glycerol, and stored at 4°C until processing. At 14 dpc, blood was collected from all chickens and at 21 dpc, chickens were humanely euthanized.

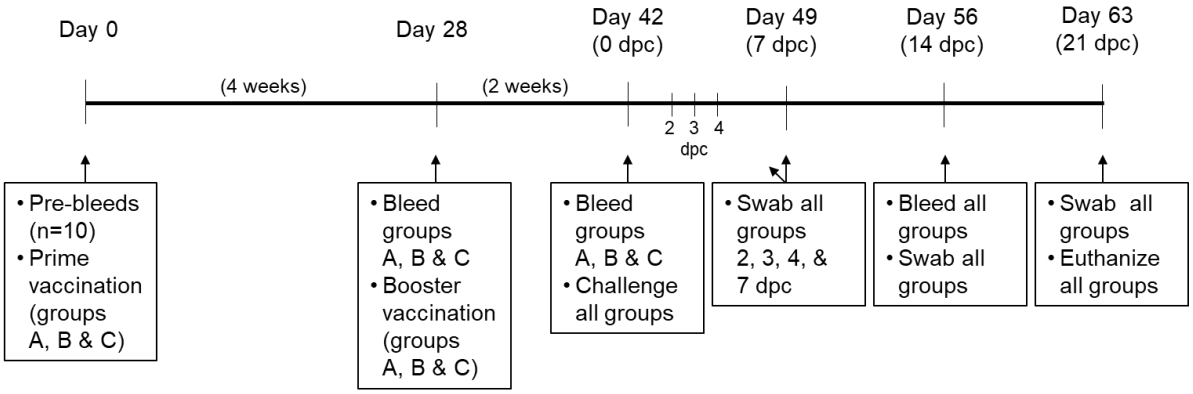


Figure 4.2. Experimental design of this study, with termination occurring at 21 days post challenge (dpc).

4.2.3.5. Serological testing

To assess humoral immune responses, blood was collected before the booster vaccination, before viral challenge, and 2 weeks after viral challenge. Blood was incubated at room temperature for at least an hour, centrifuged at 5,000 x g for 10 minutes at 4°C, and the sera transferred to sterile tubes. IDEXX Influenza A virus Ab test kits were used to detect NP-specific antibodies, which are antigenically conserved amongst influenza A viruses (OIE, 2018), according to manufacturer's instructions and the absorbance values determined using an iMark™ Microplate Reader (BioRad). The sample-to-negative control ratio (S/N) of this competitive enzyme-linked immunosorbent assay (ELISA) was calculated from the absorbance at 655 nm (A^{655}) for each sample, with a S/N value of less than 0.5 being considered as positive. Sera were also submitted to the University's Department of Veterinary Tropical Diseases, Serology Laboratory, for hemagglutination and HI testing, according to the OIE-recommended procedures (OIE, 2018). Two antigens were used for HI testing, which measures HA-specific antibodies: 1) A/chicken/South Africa/W-04/2002 (H6N2), which is homologous to the commercial H6N2 vaccine (AVIVAC® AI) and is used routinely for diagnostic purposes (Rauff *et al*., 2016); and 2) the challenge virus A/chicken/South Africa/H44954/2016 (H6N2), which is 95.8% identical to the H6 sub-lineage I VLP strain in the HA protein (Table 4.1). The H6 sub-lineage I VLPs homologous live virus, A/chicken/South Africa/N2826/2016 (H6N2), could not be used as HI test antigen because it was contaminated with a virulent Newcastle disease virus. As the Newcastle disease virus also agglutinates erythrocytes, it would prevent the accurate estimation of hemagglutinating units. A H6N2 sub-lineage II virus (homologous or closely related to the H6 sub-lineage II VLP vaccine) was not available for use at the time of the study. The H6 sub-lineage II VLP strain is more similar in the HA protein to the commercial vaccine strain (92.9%) than the challenge virus (85.7%) (Table 4.1), and higher HI titers were subsequently expected against the 2002 HI test antigen prior to viral challenge. HI titers were considered to be positive if complete inhibition of hemagglutination was observed at a sample dilution of 1/16 (2^4 or 4 $\log_2$) or more.

4.2.3.6. Viral detection by real-time quantitative reverse transcription PCR

RNA was extracted from oropharyngeal and cloacal swab fluid using TRIzol™ Reagent (Thermo Fisher Scientific™). Swab fluid (250 µl) was incubated in TRIzol™ (750 µl) for 5 minutes, whereafter chloroform (200 µl) was added to the mixture. Following 10 minute incubation, the sample was centrifuged for 15 minutes at 13,000 x rpm and the clear upper phase was mixed with an equal volume of isopropanol (600 µl). Following 10 minutes incubation, the sample was centrifuged for 10 minutes at 13,000 x rpm and all visible liquid

was discarded. The pellet was washed using 70% [v/v] ethanol (700 µl), centrifuged for 5 minutes at 13,000 x rpm and all visible liquid was discarded. The dried RNA pellet was subsequently suspended in 50 µl of nuclease-free distilled water and subjected to qRT-PCR.

To determine the viral load, a qRT-PCR assay targeting regions of the matrix gene that are conserved amongst type A viruses (Spackman *et al.,* 2003) was used (as described by Smith *et al.,* 2019). The qRT-PCR reactions were carried out on the StepOnePlus™ platform (Applied Biosystems) using a VetMax™-Plus One-Step RT-PCR kit (Applied Biosystems). To minimize possible variation due to slight differences between qRT-PCR runs, the type of sample for all four treatment groups on a specific sampling day was grouped together on a single qRT-PCR plate (for example oropharyngeal RNA, 2 dpc) and performed at the same time. Each qRT-PCR reaction consisted of the following: 3 µl RNA, 6 µl 2 x RT-PCR buffer, 0.5 µl 25 x RT-PCR enzyme mix, 0.1 µM forward and reverse primer, respectively, 0.15 µl probe (5 µM), and PCR grade water to a final volume of 12 µl. Cycling conditions entailed 1 cycle of 48^{o}C for 10 minutes, 1 cycle of 95^{o}C for 10 minutes, and 40 cycles of 95^{o}C for 15 seconds followed by 53^{o}C for 45 seconds. Serial dilutions (10^{-1} to 10^{-6}) of RNA extracted from 250 µl of viral challenge material with a titer of $10^{6.8}$ EID_{50}/ml served as positive controls and were included in duplicate in each qRT-PCR run. The standard curve generated by the positive control samples was used to extrapolate the EID_{50} viral quantity in each sample. Samples with a cycle threshold (Ct) value of less than 40 were considered to be positive for the presence of avian influenza virus.

One EID_{50} unit is the amount of virus that will infect 50% of inoculated eggs and infectivity is determined by the number of embryo mortalities. However, as H6N2 is a LPAI virus and mortalities are not expected, the calculated EID_{50} value is underestimated. To obtain a more accurate reflection of the amount of virus shed following challenge, the EID_{50} value of each sample determined with qRT-PCR was converted to a viral RNA copy number (Smith *et al.,* 2019). To achieve this, the EID_{50} value obtained with the highest dilution of RNA extracted from the challenge material that tested positive (10^{-8}) was equated to the lowest number of AIV detectable using this qRT-PCR assay, which is 1000 viral copies according to Spackman *et al.* (2003). Therefore, the 1,000 viral RNA copy number was divided by the EID_{50} value of the 10^{-8} dilution (0.019456) to obtain a factor of 51,398.03. For each sample that tested positive with qRT-PCR, the EID_{50} value was multiplied by a factor of 51,398.03 to obtain the viral RNA copy number.

4.2.3.7. Statistical analysis

Virus titers and antibody titers among groups were analyzed using One-Way analysis of variance (ANOVA). Pairwise mean comparisons between groups were analyzed using the Student *t*-test. A p-value < 0.05 was considered to be a statistically significant difference between treatment groups.

4.3. Results and discussion

4.3.1. H6 VLP production and purification

Making use of the pEAQ-HT plant expression vector and agroinfiltration, H6 sub-lineage I and II influenza VLPs containing HA and matrix 2 (M2) were transiently produced in glyco-engineered *N. benthamiana* plants (Strasser *et al.,* 2008). *A. tumefaciens* suspensions (AG-1, OD_{600} =1.5) containing equal parts H6 HA_0 (sub-lineage I and sub-lineage II, respectively) and M2 validated constructed vectors were introduced into five-to-eight-week plant leaves using a syringe without a needle. After six days, the infiltrated leaves were harvested, homogenized in two volumes of Tris buffer (Landry *et al.,* 2010), clarified and purified using differential centrifugation (20-60% Iodixanol density gradients (OptiPrep™; Sigma Aldrich)). The fractions containing the most abundant HA protein (i.e. fractions 10 to 12, which corresponds to 20 to 30% iodixanol) were pooled and dialyzed in PBS, whereafter the disaccharide Trehalose (15% w/v; Sigma-Aldrich) was added as stabilizing agent (Lynch *et al.,* 2012). Following SDS-PAGE and immunoblot analysis of the partially-purified product (Figure 4.3), TEM revealed abundant VLPs resembling native influenza viral particles for sub-lineage I, although very few sub-lineage II VLPs were observed, corresponding to the lower protein yield attained with the sub-lineage II construct. Hemagglutination and hemagglutination inhibition (HI) assays confirmed functionality. For sub-lineage I VLPs a titer per 25 µl of 9 $\log_2$ (512 HA units (HAU)) and a HI titer of 6 $\log_2$ were obtained, while a markedly lower titer per 25 µl of 3 $\log_2$ (8 HAU) and a HI titer of 4 $\log_2$ were obtained for sub-lineage II VLPs. As previously discussed, low non-specific reactions were observed with the HI assay against the negative control SPF sera, likely due to the presence of other plant proteins, and the absolute HI titer is consequently difficult to determined.

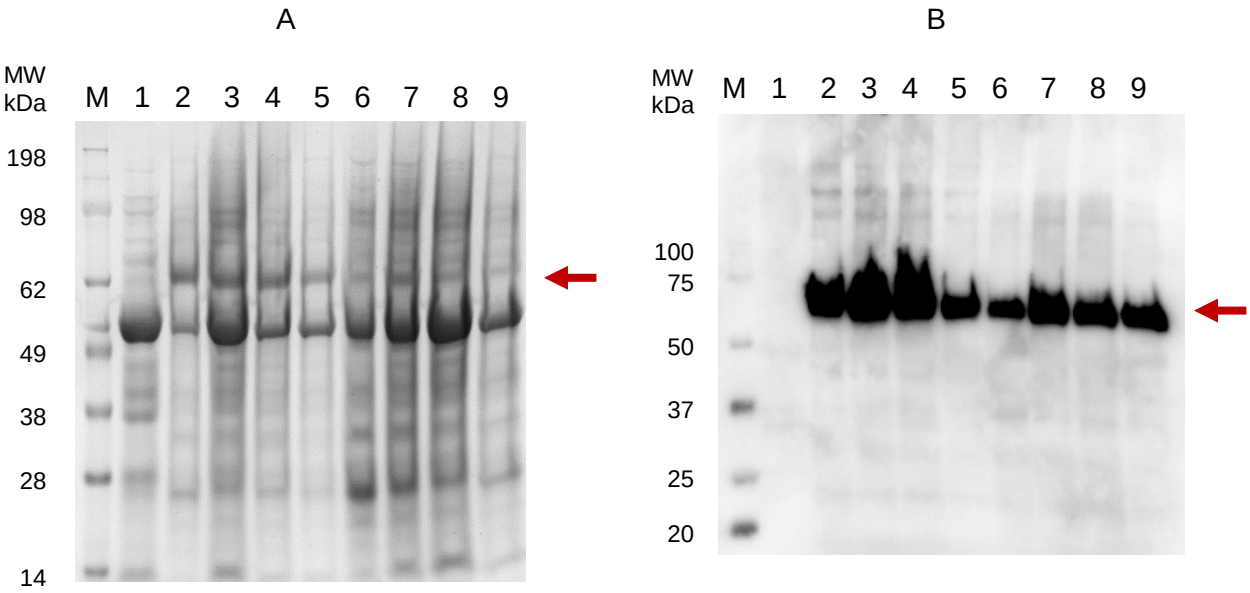


Figure 4.3. SDS-PAGE (A) and immunodetection (B) of partially-purified plant-produced H6 sub-lineage I and II hemagglutinin (HA_0). Lane 1: negative control (plant-expressed pEAQ-HT); lanes 2 to 4: H6 sub-lineage I HA present in fractions 10, 11 and 12 of the Iodixanol density gradient; lane 5: H6 sub-lineage I HA dialyzed in phosphate buffered saline (PBS) and stabilized with Trehalose dihydrate; lanes 6 to 8: H6 sub-lineage II HA present in fractions 10, 11 and 12 of the Iodixanol density gradient; lane 9: H6 sub-lineage II HA dialyzed in PBS and stabilized with Trehalose dihydrate. M: The SeeBlue Plus2 (A) and WesternC (B) protein molecular weight markers were used for the SDS-PAGE and immunoblot, respectively. The arrows indicate the position of the target protein (approximately 62 kDa).

4.3.2. Evaluation of the humoral immune responses

Prior to the start of the trial, blood was collected from 10 of the 6-week-old SPF chickens that were randomly selected and assigned to the non-vaccinated control group (Group D). Sera were subjected to testing using the IDEXX Influenza A virus Antibody test kit according to manufacturer's instructions, and the sample-to-negative control ratio (S/N) values for all 10 chickens were negative (S/N < 0.5; data not shown), confirming that the flock had not prior exposure to avian influenza. Chickens in treatment group A (plant-produced H6 sub-lineage I VLP vaccine adjuvanted with Montanide™ ISA 71 VG (Seppic, France), 769 HAU/0.3 ml dose), treatment group B (plant-produced H6 sub-lineage II VLP vaccine adjuvanted with Montanide™ ISA 71 VG (Seppic, France), 48 HAU/0.3 ml dose (the maximum antigenic dose)) and treatment group C (commercial inactivated H6N2 oil-emulsion vaccine, $EID_{50} \geq 10^8$/0.5 ml dose) were vaccinated at 6 and 10 weeks of age and challenged two weeks later with a heterologous 2016 H6N2 virus (10^6 EID_{50}/0.6ml). To evaluate the humoral immune

response with HI (HA-specific antibodies) and IDEXX ELISA (nucleoprotein (NP)-specific antibodies) (Table 4.2), blood was collected four weeks after the primary immunization (prior to administration of the booster vaccine) (groups A, B and C), two weeks after the booster immunization (prior to viral challenge) (groups A, B and C) and two weeks (14 days) after viral challenge (groups A, B, C and D). As the most accurate quantitation of HA-specific antibodies is obtained using a homologous or closely related virus (Swayne *et al.,* 2015), the challenge virus ("2016 HI test antigen", which is 95.8% identical to the H6 VLP's homologous strain in the HA protein) was used as HI test antigen in addition to the antigen used for routine testing in South Africa ("2002 HI test antigen", which is homologous with the commercial H6N2 vaccine seed strain). Due to the presence of a contaminating virulent Newcastle disease virus the H6 VLP's homologous live virus could not be used as a test antigen, while a sub-lineage II virus was not available for use. In group B, the total number of birds after the primary immunization was reduced to 11 as chicken B12 escaped its pen and got into group C's pen, and the serological (and viral shedding) results of bird B12 was consequently excluded from the study.

4.3.2.1. Antibody titers four weeks after the primary immunization

Four weeks after the primary immunization (pre-booster), high HI titers were detected in treatment group A (H6 sub-lineage I VLP vaccine) against the more closely related 2016 test antigen (geometric mean titer (GMT) of 9.3 $\log_2$, ranging from 8 $\log_2$ to 12 $\log_2$), although the HI titers were markedly lower against the 2002 test antigen (GMT of 6.1, ranging from 4 $\log_2$ to 9 $\log_2$) (Table 4.2). In treatment group B (H6 sub-lineage II VLP vaccine), the HA-specific antibody titers reflected the low antigenic dose of the vaccine, with GMTs of 3.3 $\log_2$ (ranging from 2 $\log_2$ to 5 $\log_2$) against the 2002 antigen and 2.7 $\log_2$ (ranging from 1 $\log_2$ to 5 $\log_2$) against the 2016 antigen. In treatment group C, GMTs of 7.1 $\log_2$ and 6.0 $\log_2$ were present against the homologous 2002 test antigen and the 2016 test antigen, respectively, and a greater range in HI titer was present in comparison to group A using both the 2002 test antigen (3 $\log_2$ to 10 $\log_2$) and the 2016 test antigen (2 $\log_2$ to 8 $\log_2$) (Table 4.2). Using the IDEXX Influenza A virus Antibody test kit, NP-specific antibodies was detected in 9/12 chickens vaccinated with the inactivated commercial vaccine (group C) (mean S/N ratio of 0.41), whereas NP-specific antibodies were absent in the plant-produced H6 VLP-vaccinated treatment groups A (mean S/N ratio of 0.93) and B (mean S/N ratio of 0.99), as expected, as the VLPs do not contain NP (Table 4.2).

4.3.2.2. Antibody titers two weeks after the booster immunization

Two weeks after the booster immunization, NP-specific antibodies were detected in 12/12 chickens in the inactivated commercial vaccine (group C) using the IDEXX ELISA assay (mean S/N ratio of 0.15), whereas NP-specific antibodies were absent in the plant-produced H6 VLP-vaccinated treatment groups A and B (Table 4.2). Using HI assays, pre-challenge HA-specific antibodies in the prime-boost vaccinated treatment groups had increased by 1.3 $\log_2$ to 2.5 $\log_2$. For group A, the mean pre-challenge HI titer against the 2016 and 2002 antigens were 10.7 $\log_2$ (ranging from 9 $\log_2$ to 12 $\log_2$) and 8.6 $\log_2$ (ranging from 7 $\log_2$ to 10 $\log_2$), respectively (Table 4.2). For group B, the GMT using the 2002 HI test antigen had increased to 5.3 $\log_2$ after the booster vaccination and 9/10 chickens tested positive, while the 2002 HI test antigen resulted in a lower GMT of 4.0 $\log_2$ and only 7/10 chickens tested positive for the presence of HA-specific antibodies (Table 4.2). However, as a closely related/homologous hemagglutination inhibition (HI) antigen was not available for the testing of serology samples of sub-lineage II VLP-vaccinated birds and it is, therefore, likely that the H6-specific antibody titers elicited by the sub-lineage II vaccine were understated in this study. For group C, the mean pre-challenge HI titer against the 2002 antigen was comparable to that of group A at 8.8 $\log_2$ (with a range of 6 $\log_2$ to 12 $\log_2$ within the group), whereas the GMT against the 2016 antigen was 2.7 $\log_2$ values lower at 8.0 $\log_2$ (ranging from 6 $\log_2$ to 10 $\log_2$) (Table 4.2). As the challenge virus is homologous to the 2016 HI test antigen, the HI titers against the 2016 antigen provides a better indication of the ability of the respective vaccines to prevent viral replication and consequently viral shedding upon challenge, in comparison to the 2002 antigen. In previous studies involving inactivated avian influenza vaccines in SPF chickens, a GMT ≥ 40 (> 5 $\log_2$) was associated with the prevention of oropharyngeal viral shedding in most vaccinated chickens, while a GMT ≥ 128 (≥ 7 $\log_2$) was associated with the prevention of oropharyngeal viral shedding in all vaccinated chickens, if the challenge strains were closely related to the vaccine (Swayne and Kapczynski, 2017). Making use of these parameters, the 2016 HI test antigen yielded a GMT ≥ 7 $\log_2$ in 10/12 chickens following two doses of the inactivated commercial vaccine and in 12/12 chickens following a single dose of the H6 sub-lineage I VLP vaccine, while a GMT > 5 $\log_2$ was obtained in 3/10 birds following two doses of the H6 sub-lineage II VLP vaccine (Table 4.2). Thus, based on these HI results, a reduction in viral shedding was expected in most chickens vaccinated with the commercial vaccine and only in a few of the chickens vaccinated with the sub-lineage II VLP vaccine following viral challenge, while complete inhibition of viral shedding was expected in the sub-lineage I VLP vaccine group.

4.3.2.3. Antibody titers two weeks after viral challenge

Two weeks after challenge with the live replicating 2016 H6N2 virus, NP-specific antibodies were only detected in 2/12 chickens in the H6 sub-lineage I vaccine group (group A) (Table 4.2). The HI titers of group A remained high, with GMTs of 7.3 $\log_2$ and 9.9 $\log_2$ against the 2002 and 2016 HI test antigens, respectively (Table 4.2). In group B (H6 sub-lineage II VLP vaccine), NP-specific antibodies were detected in 10/10 birds (mean S/N ratio of 0.27) and the mean HI titers had increased to 7.8 $\log_2$ and 8.5 $\log_2$ against the 2002 and 2016 HI test antigens, respectively (Table 4.2). In the commercial vaccine group (group C), NP-specific antibodies were detected in 12/12 birds (mean S/N ratio of 0.10) and the mean HI titers had increased to 7.8 $\log_2$ and 8.5 $\log_2$ against the 2002 and 2016 HI test antigens, respectively (Table 4.2). For the non-vaccinated control group (group D), NP-specific antibodies were detected in 12/12 chickens, confirming successful replication of the challenge virus, and 12/12 birds tested positive (GMT 7.9 $\log_2$) for HA-specific antibodies against the 2016 HI test antigen. Against the 2002 HI test antigen, however, which is used routinely to screen for H6N2 avian influenza, only 6/12 birds tested positive (GMT 4.2 $\log_2$), demonstrating the extent of the evolutionary changes that have occurred in H6N2 viruses since 2002. Therefore, there is an urgent need to update the HI test antigen to ensure accurate screening of flocks for the presence of H6 LPAI.

Table 4.2. Serology test results for influenza A nucleoprotein antibody ELISAs and hemagglutination inhibition (HI) assays with positive values in boldface.

Treatment group	Chicken No.	10 weeks of age 4 weeks post primary vaccination (pre-booster titers)			12 weeks of age 2 weeks post booster vaccination (pre-challenge titers)			14 weeks of age 2 weeks post challenge		
		ELISA S/N†	H6N2 HI Log_2 titer		ELISA S/N†	H6N2 HI Log_2 titer		ELISA S/N†	H6N2 HI Log_2 titer	
			2002 antigen‡	2016 antigen§		2002 antigen‡	2016 antigen§		2002 antigen‡	2016 antigen§
A: H6 Sub-lineage I VLP vaccine	A1	1.37	**4**	**8**	1.01	**8**	**10**	0.82	**5**	**7**
	A2	0.90	**7**	**10**	0.84	**10**	**11**	0.81	**7**	**9**
	A3	0.96	**6**	**9**	0.80	**7**	**9**	**0.22**	**9**	**12**
	A4	0.95	**6**	**10**	0.84	**10**	**12**	0.92	**8**	**10**
	A5	1.05	**7**	**10**	0.90	**10**	**11**	0.87	**7**	**9**
	A6	0.93	**6**	**9**	0.79	**8**	**10**	0.60	**6**	**9**
	A7	0.81	**5**	**8**	0.88	**7**	**9**	0.80	**7**	**10**
	A8	0.85	**5**	**8**	0.84	**9**	**12**	0.79	**7**	**10**
	A9	1.05	**6**	**10**	0.87	**9**	**12**	0.64	**7**	**11**
	A10	1.00	**5**	**8**	0.74	**8**	**10**	**0.43**	**9**	**12**
	A11	0.98	**7**	**12**	0.82	**8**	**11**	1.01	**6**	**8**
	A12	0.98	**9**	**10**	0.80	**9**	**11**	0.89	**9**	**12**
	GMT	**0.99 ± 0.14**	**6.1 ± 1.3**	**9.3 ± 1.2**	**0.84 ± 0.07**	**8.6 ± 1.1**	**10.7 ± 1.1**	**0.73 ± 0.23**	**7.3 ± 1.3**	**9.9 ± 1.6**
B: H6 Sub-lineage II VLP vaccine	B1	0.98	**5**	**5**	0.84	**7**	**6**	**0.27**	**8**	**9**
	B2	0.90	**4**	3	0.78	**6**	**4**	**0.25**	**9**	**9**
	B3	1.05	3	1	0.86	**6**	**4**	**0.35**	**7**	**9**
	B4	0.82	3	3	0.87	**6**	**5**	**0.23**	**9**	**10**
	B5	1.16	2	1	0.86	2	1	**0.34**	**7**	**7**
	B6	0.69	**4**	**5**				**¥**		
	B7	0.99	3	1	0.95	**4**	2	**0.19**	**9**	**9**
	B8	0.89	**4**	3	0.83	**7**	**6**	**0.11**	**10**	**9**
	B9	0.94	2	2	0.86	**5**	**4**	**0.21**	**7**	**8**
	B10	0.88	2	1	0.89	**4**	2	**0.39**	**6**	**8**
	B11	0.91	**4**	**5**	0.95	**6**	**6**	**0.40**	**6**	**7**
	GMT	**0.93 ± 0.12**	**3.3 ± 1.0**	**2.7 ± 1.7**	**0.87 ± 0.05**	**5.3 ± 1.6**	**4.0 ± 1.8**	**0.27 ± 0.09**	**7.8 ± 1.4**	**8.5 ± 0.97**
C: Commercial H6N2 vaccine	C1	0.90	**6**	**4**	**0.17**	**9**	**8**	**0.11**	**10**	**9**
	C2	0.64	**8**	**7**	**0.14**	**9**	**9**	**0.13**	**8**	**9**
	C3	**0.26**	**9**	**8**	**0.14**	**10**	**9**	**0.14**	**7**	**7**
	C4	**0.48**	**4**	2	**0.11**	**7**	**6**	**0.08**	**6**	**8**
	C5	**0.14**	**6**	**5**	**0.15**	**8**	**7**	**0.08**	**9**	**10**
	C6	**0.07**	**9**	**8**	**0.07**	**10**	**8**	**0.05**	**10**	**11**
	C7	**0.46**	**8**	**8**	**0.16**	**9**	**9**	**0.30**	**9**	**7**
	C8	**0.09**	**10**	**8**	**0.15**	**12**	**10**	**0.05**	**8**	**8**
	C9	**0.42**	**7**	**6**	**0.27**	**9**	**8**	**0.07**	**8**	**9**
	C10	1.07	3	2	**0.20**	**6**	**6**	**0.07**	**7**	**9**
	C11	**0.30**	**8**	**7**	**0.17**	**7**	**7**	**0.05**	**7**	**9**
	C12	**0.13**	**7**	**7**	**0.07**	**10**	**9**	**0.04**	**8**	**9**
	GMT	**0.41 ± 0.32**	**7.1 ± 2.1**	**6.0 ± 2.3**	**0.2 ± 0.1**	**8.8 ± 1.6**	**8.0 ± 1.3**	**0.1 ± 0.1**	**8.1 ± 1.2**	**8.8 ± 1.1**
D: Non-vaccinated control	D1	nt	nt	nt	nt	nt	nt	**0.31**	**5**	**8**
	D2	nt	nt	nt	nt	nt	nt	**0.15**	**5**	**8**
	D3	nt	nt	nt	nt	nt	nt	**0.24**	3	**8**
	D4	nt	nt	nt	nt	nt	nt	**0.27**	**6**	**8**
	D5	nt	nt	nt	nt	nt	nt	**0.39**	**6**	**8**
	D6	nt	nt	nt	nt	nt	nt	**0.48**	3	**6**
	D7	nt	nt	nt	nt	nt	nt	**0.28**	3	**7**
	D8	nt	nt	nt	nt	nt	nt	**0.20**	3	**8**
	D9	nt	nt	nt	nt	nt	nt	**0.29**	**5**	**9**
	D10	nt	nt	nt	nt	nt	nt	**0.18**	**5**	**9**
	D11	nt	nt	nt	nt	nt	nt	**0.25**	3	**8**
	D12	nt	nt	nt	nt	nt	nt	**0.20**	3	**8**
	GMT							**0.27 ± 0.1**	**4.2 ± 1.3**	**7.9 ± 0.8**

†Sample to negative ratio; ‡A/chicken/South Africa/W-04/2002 (H6N2) antigen; §A/chicken/South Africa/H44954/2016 (H6N2) antigen; ¥Bird was injured and euthanized for humane reasons; nt - samples not collected for testing; GMT- geometric mean titer.

4.3.3. Evaluation of viral shedding following challenge

As the main determinant of vaccine efficacy is its ability to prevent or reduce viral shedding, swabs were collected from the oropharynx and cloaca, respectively, at 2, 3, 4, 7, 14 and 21 dpc and extracted nucleic acids were tested for the presence of the influenza A matrix gene using qRT-PCR. However, as H6N2 is a LPAI virus and mortalities of infected embryos are not expected, the EID_{50} value of each sample determined with qRT-PCR was converted to a viral RNA copy number to obtain a more accurate reflection of the quantities of virus shed following challenge. This viral RNA copy number (vRNA copies/ml) is based on the lowest limit of detection for the qRT-PCR assay used in this study (i.e. a 1,000 viral copies), as determined by Spackman *et al.,* (2003). As EID_{50}/ml is the standard unit of measurement employed to determine viral load, the EID_{50} values were used to determine the overall reduction (or increase) in viral shedding of each vaccinated treatment group in comparison to the non-vaccinated control group. The total number of birds in group B (H6 sub-lineage II VLP vaccine) was further reduced to 10 after viral challenge, as chicken B6 was euthanized on the day of viral challenge due to humane reasons unrelated to viral challenge. In group D, chicken D2 was euthanized at 16 dpc due to humane reasons unrelated to viral challenge, yielding a total number of 11 birds in the non-vaccinated group at 21 dpc.

4.4.3.1. Oropharyngeal viral shedding

In the H6 sub-lineage I VLP-vaccinated group (group A), 7/12 chickens were actively shedding virus from the respiratory tract at 3 dpc with a mean group titer of 3.49 $\log_{10}$ vRNA copies/ml (Figure 4.4, Table 4.3). The highest amounts of virus were detected from the oropharyngeal swabs of individuals A3 and A10 at 9.36 $\log_{10}$ and 9.28 $\log_{10}$ vRNA copies/ml, respectively. At 7 dpc, the viral shedding had ceased except for birds A2 (6.23 $\log_{10}$ vRNA copies/ml), A3 (7.30 $\log_{10}$ vRNA copies/ml) and A10 (7.54 $\log_{10}$ vRNA copies/ml), with a group mean of 1.76 $\log_{10}$ vRNA copies/ml (Figure 4.4, Table 4.3). At day 14 post challenge, only a single bird (namely A2) was shedding virus from the respiratory tract, at the reduced level of 3.32 $\log_{10}$ vRNA copies/ml (Figure 4.4, Table 4.3). The H6 sub-lineage I VLP vaccine resulted in a significant reduction ($p< 0.05$) in oropharyngeal shedding from day 2 to 14 post challenge (at which point shedding ceased). Individuals A3 and A10 were the only two birds in the group that had positive NP ELISA results at 14 dpc (S/N ratio of 0.22 and 0.43, respectively), which correlates with the proportionately higher replication of challenge virus in these birds on days 2 to 7 post challenge (Table 4.3). One chicken (A7) had no detectable levels of virus in oropharyngeal swabs, and an additional two chickens (A1 and A6) had no detectable levels of virus taken from 3 dpc onwards. Since their pre-challenge antibody titers

were among the lowest in the group at 9 or 10 log_2, it was presumed that the earlier cessation of viral shedding was due to non-evaluated cellular immune responses.

In the H6 sub-lineage II VLP-vaccinated group (group B), 10/10 chickens were actively shedding virus from the oropharynx until 4 dpc (Figure 4.4, Table 4.3). Individual B4, which had the highest overall viral titer after B3, was the only bird in the group where the viral titer had increased from day 2 post challenge (9.87 log_{10} vRNA copies/ml) to day 3 post challenge (9.94 log_{10} vRNA copies/ml). At 4 dpc, individuals B3 and B4 shed the highest amounts of virus at 9.73 log_{10} and 9.94 log_{10} vRNA copies/ml, respectively, with a group mean of 8.98 log_{10} vRNA copies/ml. The proportion of shedders had reduced to 6/10 at 7 dpc (mean 2.65 log_{10} vRNA copies/ml) and to 3/10 at 14 dpc (mean 1.08 log_{10} vRNA copies/ml). For birds B5 and B10, which had some of the lowest HI titers pre-challenge (1 log_2 and 2 log_2 against the 2016 HI antigen, respectively), viral shedding fell below the limit of detection from 14 dpc onwards. At 21 dpc, 3/10 chickens (B3, B7 and B8) were still shedding detectable quantities of virus from the respiratory tract, with a mean titer of 1.09 log_{10} vRNA copies/ml (Figure 4.4, Table 4.3). The H6 sub-lineage II VLP vaccine resulted in a significant ($p < 0.5$) reduction in oropharyngeal viral shedding titer at 7 dpc and 14 dpc in comparison to the commercial vaccine group C, but not the non-vaccinated control group D (Figure 4.4). All 10 birds tested with positive with the NP ELISA at 14 dpc (Table 4.3).

In contrast to the sub-lineage I and II VLP vaccines, a larger proportion of chickens vaccinated with the commercial vaccine (group C) or non-vaccinated (group D) shed markedly more virus from the oropharynx, for a longer period. At 7 dpc, 12/12 chickens in group C and 11/12 chickens in group D shed high quantities of viruses, with mean viral titers of 6.96 log_{10} and 4.87 log_{10} vRNA copies/ml, respectively (Figure 4.4, Table 4.3). At 14 dpc, the proportion of shedders in groups C and D had reduced to 9/12 (mean 3.57 log_{10} vRNA copies/ml) and 6/12 (mean 1.96 log_{10} vRNA copies/ml), respectively (Figure 4.4, Table 4.3). At 21 dpc when the study ended, 7/12 chickens vaccinated with the commercial vaccine (group B) shed detectable levels of virus from the respiratory tract (mean 3.88 log_{10} vRNA copies/ml), in comparison to a lower proportion of shedders in the non-vaccinated control group D (4/11 shedders, mean 3. 78 log_{10} vRNA copies/ml), the H6 sub-lineage I VLP vaccine group A (0/12 shedders) as well as the H6 sub-lineage II VLP vaccine group B (3/10 shedders, mean 3. 64 log_{10} vRNA copies/ml) (Figure 3A, Table 4.3). A statistically significant ($p > 0.5$) increase in mean oropharyngeal viral titers of group C in comparison to the non-vaccinated control group D was determined at 7 dpc (Figure 4.4). All 12 birds tested with positive with the NP ELISA at 14 dpc, as expected (Table 4.3).

To determine the reduction in viral shedding between treatment groups, the mean EID_{50}/ml viral titers of the groups were compared. The total mean EID_{50}/ml viral titers days 2 to 21 post challenge amounted to 9,882.14 for group A, 183,944.61 for group B, 1,038,320.84 for group C and 992,697.26 for group D (Table 4.3, Table 4.4). Interestingly, it appears that whereas the commercial vaccine initially reduced the mean post-challenge oropharyngeal viral shed titers by 16.33% at 2 dpc, it augmented viral shedding by 4.39% overall in comparison to the non-vaccinated group (Table 4.4). Overall, the H6 sub-lineage I VLP vaccine resulted in a reduction of more than 100-fold in comparison to the non-vaccinated group (a 99.00% reduction) and the commercial vaccine (a 99.05% reduction), respectively (Figure 4.4). The H6 sub-lineage II VLP vaccine resulted in a modest reduction in mean oropharyngeal shedding of 5.4-fold in comparison to the non-vaccinated group C (a 81.47% reduction) and 5.64-fold in comparison to the commercial vaccine group D (a 82.28% reduction) (Table 4.4). For the sub-lineage II VLP vaccine, the results were drastically different when the total of the individual viral titers in EID_{50}/ml (days 2 to 21 post challenge) was used to determine the reduction in shedding instead of the group mean, yielding a reduction in titers of more than 60-fold in comparison to both the non-vaccinated group (a 98.46% reduction) and commercial vaccine (a 98.52% reduction) (Table 4.4). Therefore, it is suspected that an increase in the antigenic mass dose of the sub-lineage II VLP vaccine could lead to a greater reduction in viral shedding titers and the proportion of shedders, as well as shorten the duration of shedding in vaccinated SPF chickens.

4.4.3.2. Cloacal viral shedding

The total mean oropharyngeal shedding for each treatment group was between 5.09- and 6.51-fold higher compared to cloacal viral titers in log_{10} terms (calculated from EID_{50} values in Tables 4.4 and 4.6), which is consistent with other published studies for LPAI in chickens (Morales *et al*., 2009; Arafat *et al*., 2018). However, the numerical proportion in EID_{50}'s is orders of magnitude greater, with a more than 5.7 million-fold difference between oropharyngeal and cloacal shedding determined 2 days post challenge in the non-vaccinated control group D (as assessed from Tables 4.4 and 4.6). Cloacal shedding from the sub-lineage I H6 VLP-vaccinated group A was only detected in the first 7 days, whereas 1/10 birds vaccinated with the sub-lineage II H6 VLPs were still shedding from the cloaca at 14 dpc (3. 33 log_{10} vRNA copies/ml or 0.04 EID_{50}/ml) (Table 4.5). No virus was detected in the cloacal swabs of any group at 21dpc.

In the H6 sub-lineage I VLP-vaccinated group (group A), 5/12 chickens were shedding with a group mean of 1.52 log_{10} vRNA copies/ml at 2 dpc, but the proportion of shedders dropped

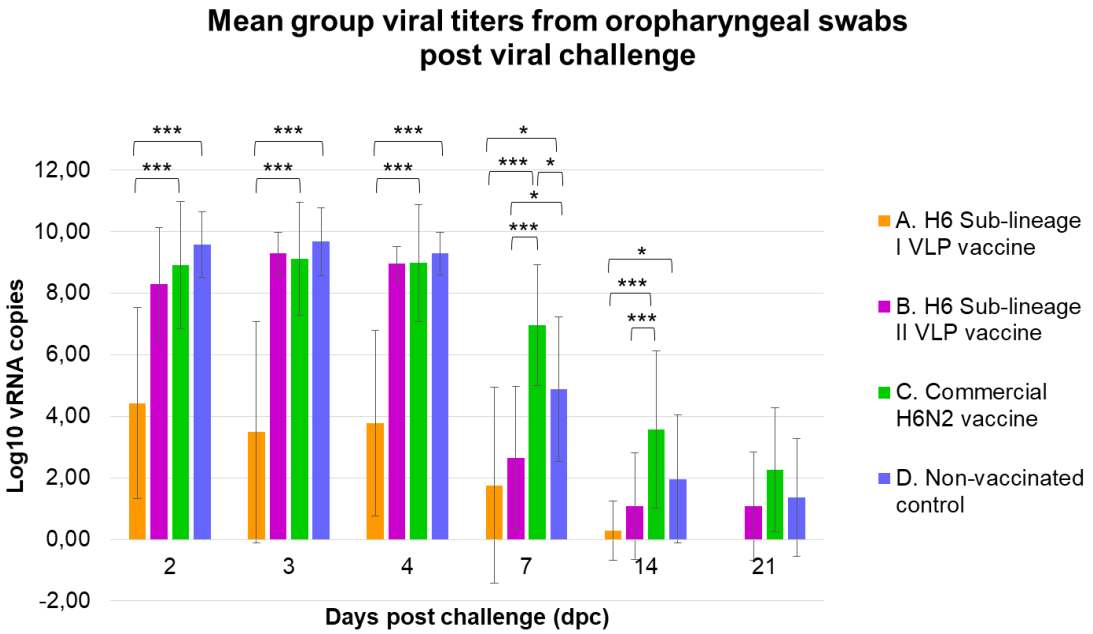
to 1/12 at 3 dpc with a group mean of 0.29 log_{10} vRNA copies/ml (Table 4.5, Table 4.6). At 4 dpc 2/12 birds were shedding detectable levels of virus from the cloaca (group mean titers of 0.60) and at 7 dpc, virus was detected from the swabs of three birds, namely A3 (3.46 log_{10} vRNA copies/ml), A10 (3.07 log_{10} vRNA copies/ml) and A11 (3.09 log_{10} vRNA copies/ml), respectively, with a group mean of 0.80 log_{10} vRNA copies/ml. Shedding had ceased completely by 14 dpc (Table 4.5, Table 4.6). Overall, in the first four days post challenge, cloacal shedding in the sub-lineage I VLP-vaccinated group was reduced (descriptively at day 2 and statistically at days 3 and 4; $p < 0.05$) compared to the non-vaccinated control group D (Table 4.6). The total mean EID_{50}/ml cloacal viral titers of the sub-lineage I vaccine group were 7.11-fold lower (85.93% reduction) in comparison to the non-vaccinated control group D and 4.43-fold lower (77.44% reduction) in comparison to the commercial vaccine group C.

In the H6 sub-lineage II VLP-vaccinated group (group B), 10/10 of the H6 VLP-vaccinated chickens were shedding at 2 dpc (mean of 3.85 log_{10} vRNA copies/ml), but the proportion of shedders dropped to 7/10 at 3 dpc with a group mean of 2.66 log_{10} vRNA copies/ml (Table 4.5, Table 4.6). At 4 dpc 10/10 birds were shedding detectable levels of virus from the cloaca (group mean titers of 3.86 log_{10} vRNA copies/ml) and at 7 dpc virus were detected from a single bird, namely B10 (3.31 log_{10} vRNA copies/ml. Shedding had ceased completely by 21 dpc, with only a single bird actively shedding virus from the gastrointestinal tract (3.33 log_{10} vRNA copies/ml) at 14 dpc (Table 4.5). Overall, mean cloacal shedding in the sub-lineage II VLP-vaccinated group was higher in comparison to the non-vaccinated (0.81-fold increase) and commercial vaccine control groups (0.51-fold increase), with statistically significant ($p < 0.05$) increases at days 2 and 4 post challenge (Table 4.6). As with the oropharyngeal swabs, the results were drastically different for the sub-lineage II VLP vaccine when the total of the individual cloacal viral titers in EID_{50}/ml (days 2 to 21 post challenge) were used to determine the reduction in shedding instead of the group mean, yielding a reduction in titers of more than 8-fold in comparison to the non-vaccinated group (a 97.56% reduction) and more than 5-fold compared to the commercial vaccine (a 80.52% reduction) (Table 4.6).

In the commercial H6N2 inactivated vaccine group (group C), viral shedding was detectable from the cloaca until 7 dpc, although the number of birds shedding (10/12, 5/12 and 7/12 for days 2, 3 and 4 post challenge, respectively) was considerably higher in comparison to group A (5/12, 1/12 and 2/12 for days 2, 3 and 4 post challenge, respectively), with an increase in viral titers ranging between 2.5- and 19.4-fold during this period (Table 4.5, Table 4,6). In group C, shedding was detectable until 7 dpc in 3/12 chickens with a group average of 0.9

log_{10} vRNA copies/ml, slightly higher than group A (0.8 log_{10} vRNA copies/ml). Cloacal viral titers in group C was detectable in 2/12 birds at 14 dpc, with viral titers of 3.42 log_{10} and 3.43 log_{10} vRNA copies/ml in birds D3 and D5, respectively, and a group mean titer of 0.57 log_{10} vRNA copies/ml (Table 4.5, Table 4.6).

A



B

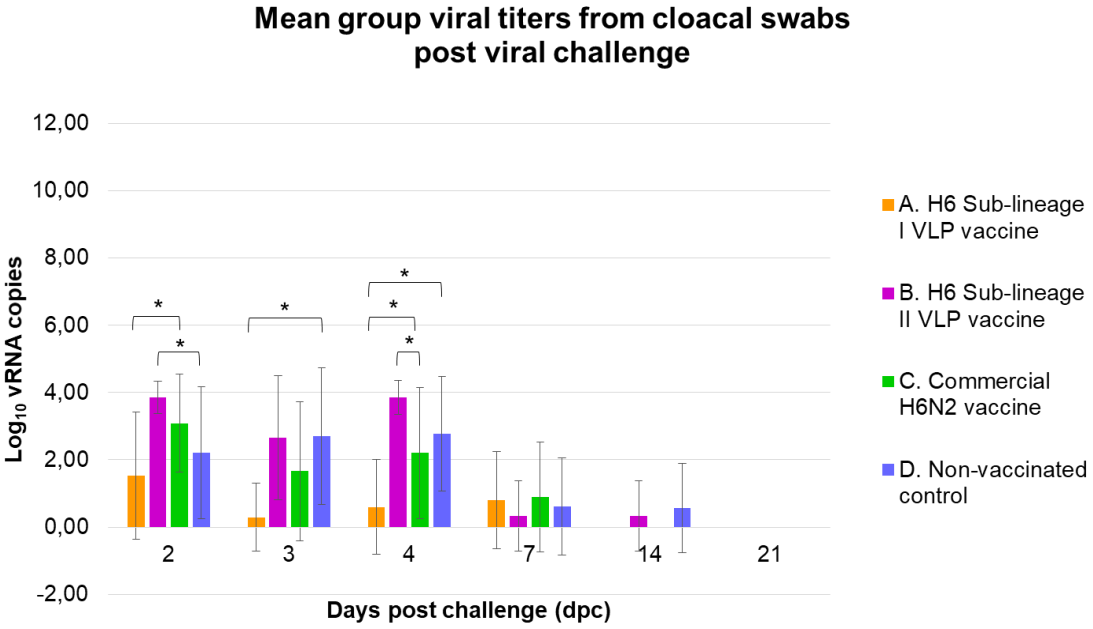


Figure 4.4. qRT-PCR results for oropharyngeal swabs (A) and cloacal swabs (B). For each group, the average log_{10} viral RNA (vRNA) viral titers/ml ± the standard deviations of the mean are indicated for swabs collected 2, 3, 4, 7, 14 and 21 days following viral challenge (dpc). The symbols *(p<0.05) and *** (p<0.001) indicate statistical differences (Student *t*-test) between two groups at a specific time point.

Table 4.3. Individual qRT-PCR results for oropharynageal swabs. Log_{10} vRNA viral titers/ml are indicated with EID_{50}/ml titers in parenthesis, as well as the total individual viral loads (EID_{50}/ml).

Group	Chicken No	Sampling day post challenge						(Total viral load in EID_{50}/ml)
		2	3	4	7	14	21	
A. H6 sub-lineage I VLP vaccine	A1	3.40 [0.05]	U	U	U	U	U	(0.05)
	A2	6.80 [122.80]	6.65 [87.89]	6.63 [83.08]	6.23 [33.05]	3.32 [0.04]	U	(326.87)
	A3	8.56 [7,087.50]	9.36 [44,166.00]	8.49 [5,972.72]	7.30 [390.38]	U	U	(57,616.60)
	A4	3.74 [0.11]	3.65 [0.09]	4.07 [0.23]	U	U	U	(0.42)
	A5	U	5.48 [5.91]	3.24 [0.03]	U	U	U	(5.94)
	A6	4.51 [0.63]	U	U	U	U	U	(0.63)
	A7	U	U	U	U	U	U	(U)
	A8	U	3.68 [0.09]	3.65 [0.09]	U	U	U	(0.18)
	A9	5.80 [12.29]	3.75 [0.11]	3.44 [0.05]	U	U	U	(12.46)
	A10	8.48 [5,882.91]	9.28 [36,683.59]	8.95 [17,352.63]	7.54 [669.72]	U	U	(60,588.85)
	A11	5.93 [16.57]	U	3.32 [0.04]	U	U	U	(16.61)
	A12	5.94 [17.02]	U	3.52 [0.06]	U	U	U	(17.08)
	Mean	**4.43±3.10 [1,094.99± 2,531.06]**	**3.49±3.60 [6,745.31± 15,812.36]**	**3.78±3.03 [1,950.75± 5,144.54]**	**1.76±3.19 [91.10± 213.72]**	**0.28±0.96 [0.0034± 0.01]**	**U**	**(118,585.69)**
B. H6 sub-lineage II VLP vaccine	B1	8.68 [9,387.94]	9.21 [31,495.91]	9.01 [20,058.98]	U	U	U	(60,942.83)
	B2	6.87 [144.14]	8.19 [3,007.17]	8.09 [2,386.13]	3.52 [0.06]	U	U	(5,537.50)
	B3	10.06 [221,077.73]	10.06 [223,854.41]	9.73 [105,456.08]	U	3.94 [0.17]	3.62 [0.08]	(550,388.47)
	B4	9.52 [64,003.78]	9.87 [142,640.84]	9.94 [168,919.22]	5.11 [2.52]	U	U	(375,566.36)
	B5	9.31 [39,307.30]	9.86 [140,451.19]	8.99 [18,893.96]	4.78 [1.19]	U	U	(198,653.64)
	B7	9.57 [72,545.61]]	9.87 [145,719.67]	9.04 [21,565.99]	U	3.38 [0.05]	3.80 [0.12]	(239,831.44)
	B8	4.86 [1.41]	8.57 [7,180.08]	8.49 [6,019.08]	4.26 [0.35]	U	3.49 [0.06]	(13,200.99)
	B9	10.04 [212,747.16]	9.71 [99,732.01]	9.04 [21,117.81]	4.85 [1.38]	3.48 [0.06]	U	(333,598.41)
	B10	5.74 [10.68]	9.20 [30,613.81]	8.89 [15,087.34]	4.02 [0.20]	U	U	(45,712.04)
	B11	8.21 [3,163.16]	8.50 [6,186.50]	8.53 [6,664.73]	U	U	U	(16,014.39)
	Mean	**8.29±1.85 [62,238.89± 85,895.60]**	**9.30±0.68 [83,088.16± 77,714.64]**	**8.98±0.55 [38,616.93± 54,494.02]**	**2.65±2.33 [0.57± 0.85]**	**1.08±1.74 [0.03± 0.05]**	**1.09 ±1.76 [0.03± 0.04]**	**(183,945.11)**

(Table 4.3 continued)

Group	Chicken No	Sampling day post challenge						(Total viral load in EID_{50}/ml)
		2	3	4	7	14	21	
C. Commercial H6N2 vaccine	C1	8.95 [17,147.34]	9.20 [31,123.68]	9.47 [56,762.66]	7.82 [1,271.09]	4.07 [0.23]	3.30 [0.04]	(106,305.04)
	C2	9.21 [31,693.99]	9.03 [20,857.23]	8.98 [18,794.16]	5.67 [9.15]	4.66 [0.89]	U	(71,355.42)
	C3	4.44 [0.54]	5.47 [5.72]	5.68 [9.35]	3.96 [0.18]	U	U	(15.79)
	C4	8.69 [9,496.08]	10.06 [222,982.17]	10.38 [465,251.66]	7.35 [436.86]	5.20 [3.08]	3.82 [0.13]	(698,169.98)
	C5	10.18 [294,594.91]	10.72 [1,011,907.56]	10.02 [204,423.14]	9.29 [38,369.80]	3.51 [0.06]	U	(1,549,295.47)
	C6	5.38 [4.67]	5.57 [7.21]	4.80 [1.22]	3.63 [0.08]	U	U	(13.19)
	C7	8.40 [4,941.30]	8.33 [4,172.49]	7.97 [1,808.42]	5.09 [2.38]	3.78 [0.12]	3.77 [0.11]	(10,924.83)
	C8	9.68 [94,055.52]	9.29 [38,088.14]	10.53 [653,765.94]	9.25 [34375.70]	8.87 [14,506.68]	U	(834,791.97)
	C9	11.26 [3,554,684.50]	10.47 [573,779.94]	9.41 [49,937.49]	7.49 [607.32]	3.82 [0.13]	4.43 [0.53]	(4,179,009.91)
	C10	10.83 [1,319,418.75]	10.73 [1,053,922.00]	10.51 [631,874.00]	8.46 [5,616.02]	4.71 [1.00]	3.99 [0.19]	(3,010,831.96)
	C11	9.71 [100,821.03]	9.86 [141,862.56]	9.67 [90,377.52]	6.73 [105.34]	U	3.55 [0.07]	(333,166.52)
	C12	10.25 [342,713.22]	10.63 [838,345.88]	10.39 [47,4669.69]	8.72 [10,240.57]	4.22 [0.32]	4.26 [0.36]	(1,665,970.03)
	Mean	**8.92±2.06 [480,797.65± 1,036,923.51]**	**9,11±1.84 [328,087.88± 420,797.26]**	**8.98±1.91 [220,639.60± 259,421.07]**	**6.96±1.96 [7,586.21± 13,823.30]**	**3.57±2.56 [1,209.38± 4,187.56]**	**3.88±0.39 0.20±0.18]**	**(12,459,850.13)**
D. Non-vaccinated control	D1	9.83 [132,184.05]	9.92 [162,635.92]	9.20 [30,582.46]	5.78 [11.67]	3.76 [0.11]	U	(325,414.21)
	D2	9.07 [22,873.56]	10.12 [255,302.47]	8.99 [19,051.86]	U	U	*	(297,227.88)
	D3	11.03 [2,094,932.25]	10.73 [1,052,160.00]	10.80 [1,229,791.88]	9.61 [79,515.58]	4.75 [1.08]	U	(4,456,400.79)
	D4	10.83 [1,308,116.13]	10.27 [366,290.41]	9.69 [95,887.45]	7.33 [416.87]	3.65 [0.09]	3.68 [0.09]	(1,770,711.03)
	D5	9.06 [22,264.40]	9.57 [72,389.12]	9.69 [96,366.26]	5.31 [4.00]	4.05 [0.22]	4.26 [0.36]	(191,024.35)
	D6	11.17 [2,880,247.00]	10.04 [212,548.03]	9.58 [74,106.70]	4.52 [0.64]	U	U	(3,166,902.38)
	D7	9.18 [29,331.09]	9.57 [72,102.30]	9.30 [39,215.80]	3.63 [0.08]	U	U	(140,649.28)
	D8	8.40 [4,852.83]	9.39 [47,933.50]	8.66 [8,931.28]	3.70 [0.10]	U	U	(61,717.71)
	D9	9.09 [23,994.57]	6.42 [50.74]	8.32 [4,092.66]	4.01 [0.20]	U	U	(28,138.18)
	D10	10.24 [335,462.56]	9.61 [79,864.50]	8.50 [6,085.93]	4.00 [0.19]	3.33 [0.04]	3.65 [0.09]	(421,413.31)
	D11	9.31 [40,149.06]	10.03 [208,241.86]	8.91 [15,671.43]	4.19 [0.30]	4.02 [0.20]	3.51 [0.06]	(264,062.91)
	D12	7.71 [1,002.83]	10.53 [654,263.75]	9.84 [133,386.89]	6.42 [51.62]	U	U	(788,705.09)
	Mean	**9.58±1.07 [574,617.53± 980,148.75]**	**9.68±1.11 [265,315.22± 305,085.74]**	**9.29±0.69 [146,097.55± 343,938.97]**	**4.87±2.35 [6,666.77± 22,941.73]**	**1.96±2.08 [0.15± 0.31]**	**3.78±0.33 [0.15± 0.14]**	**(11,912,367.11)**

U-undetected/below the limit of detection of 1000 viral copies

* chicken D2 was euthanized 16 days post challenge for humane reasons unrelated to viral challenge

Table 4.4. Mean group qRT-PCR results with standard deviation for oropharyngeal swabs. EID_{50}/ml viral titers are indicated with the proportion of shedders in parenthesis.

Description	Group(s)	Sampling day post challenge						Total mean group titers (EID_{50}/ml)	Total individual titers (EID_{50}/ml)
		2	3	4	7	14	21		
Group EID_{50}/ml titers	A	1,094.99† (9/12)‡	6,745.31† (7/12)‡	1,950.75† (9/12)‡	91.10† (3/12)‡	0.0034† (1/12)‡	U	9,882.14†	118,585.69¥
	B	62,238.89† (10/10)‡	83,088.16† (10/10)‡	38,616.93† (10/10)‡	0.57† (6/10)‡	0.03† (3/10)‡	0.03† (3/10)‡	183,944.61†	183,945.11¥
	C	480,797.65† (12/12)‡	328,087.88† (12/12)‡	220,639.60† (12/12)‡	7,586.21† (12/12)‡	1,209.38† (9/12)‡	0.12† (7/12)‡	1,038,320.84†	12,459,850.13¥
	D	574,617.53† (12/12)‡	265,315.22† (12/12)‡	146,097.55† (12/12)‡	6,666.77† (11/12)‡	0.15† (6/12)‡	0.05† (4/11)‡	992,697.26†	11,912,367.11¥
Comparison between groups	A vs. C (C/A)	439.09 X§ (A -99.77%)¶	48.64 X§ (A:-97.94%)¶	113.11 X§ (A:-99.12%)¶	83.28 X§ (A:-98.80%)¶	354,127.19 X§ (A:-100%)¶	-	105.07 X§ (A:-99.05%)¶	105.07 X§ (A:-99.05%)¶
	A vs. D (D/A)	524.77 X§ (A:-99.81%)¶	39.33 X§ (A:-97.46%)¶	74.89 X§ (A:-98.66%)¶	73.18 X§ (A:-98.63%)¶	42.59 X§ (A:- 97.65%)¶	-	100.45 X§ (A:-99.00%)¶	100.45 X§ (A:-99.00%)¶
	B vs. C (C/B)	7.73 X§ (B:-87.06%)¶	3.95 X§ (B:-74.68%)¶	5.71 X§ (B:-82.50%)¶	13,310.60 X§ (B:-99.99%)¶	40,298.32 X§ (B:-100%)¶	4.52 X§ (B:-77.88%)¶	5.64 X§ (B:-82.28%)¶	67.74 X§ (B:-98.52%)¶
	B vs. D (D/B)	9.23 X§ (B:-89.17%)¶	3.19 X§ (B:-68.68%)¶	3.78 X§ (B:-73.57%)¶	11,697.38 X§ (B:-99.99%)¶	5.33 X§ (B:-81.23%)¶	2.07 X§ (B:-51.65%)¶	5.40 X§ (B:-81.47%)¶	64.76 X§ (B:-98.46%)¶
	C vs. D (D/C)	1.20 X§ (C:-16.33%)¶	0.81 X§ (C:+19.13%)¶	0.66 X§ (C:+33.78%)¶	0.88 X§ (C:+12.12%)¶	0.00012 X§ (C:+99.99%)¶	0.46 X§ (C:+54.26%)¶	0.96 X§ (C:+4.39%)¶	0.96 X§ (C:+4.39%)¶

† Mean viral titer (EID_{50}/ml); ‡ Positive birds; § X: Fold difference in viral titers; ¶ Percentage reduction (-) or increase (+) in viral titers in comparison to the control group; ¥ Sum of the individual viral titers (EID_{50}/ml) in a treatment group.

Table 4.5. Individual qRT-PCR results for cloacal swabs. Log_{10} vRNA viral titers/ml are indicated with EID_{50}/ml titers in parenthesis, as well as the total individual viral loads (EID_{50}/ml).

Group	Chicken No.	Sampling day post challenge						(Total viral load in EID_{50}/ml)
		2	3	4	7	14	21	
A: H6 Sub-lineage I VLP vaccine	A1	3.85 [0.14]	3.51 [0.06]	U	U	U	U	(0.20)
	A2	U	U	U	U	U	U	(U)
	A3	U	U	3.92 [0.16]	3.46 [0.06]	U	U	(0.22)
	A4	U	U	U	U	U	U	(U)
	A5	3.37 [0.05]	U	3.29 [0.04]	U	U	U	(0.08)
	A6	3.68 [0.09]	U	U	U	U	U	(0.09)
	A7	3.48 [0.06]	U	U	U	U	U	(0.06)
	A8	3.90 [0.15]	U	U	U	U	U	(0.15)
	A9	U	U	U	U	U	U	(U)
	A10	U	U	U	3.07 [0.02]	U	U	(0.02)
	A11	U	U	U	3.09 [0.02]	U	U	(0.02)
	A12	U	U	U	U	U	U	(U)
	Mean	**1.52±1.89 [0.04±0.06]**	**0.29±1.01 [0.01± 0.02]**	**0.60±1.41 [0.02± 0.05]**	**0.80±1.45 [0.01± 0.02]**	**U**	**U**	**(0.86)**
B: H6 Sub-lineage II VLP vaccine	B1	3.39 [0.05]	3.73 [0.10]	3.38 [0.05]	U	U	U	(0.20)
	B2	3.41 [0.05]	3.72 [0.10]	3.65 [0.09]	U	U	U	(0.24)
	B3	3.86 [0.14]	3.69 [0.10]	3.68 [0.09]	U	U	U	(0.33)
	B4	4.78 [1.18]	U	4.78 [1.18]	U	U	U	(2.35)
	B5	3.64 [0.09]	3.75 [0.11]	4.28 [0.37]	U	U	U	(0.57)
	B7	3.20 [0.03]	3.54 [0.07]	4.18 [0.29]	U	U	U	(0.39)
	B8	3.96 [0.18]	3.99 [0.19]	3.75 [0.11]	U	3.33 [0.04]	U	(0.52)
	B9	3.91 [0.16]	U	3.40 [0.05]	U	U	U	(0.21)
	B10	4,35 [0.44]	4.21 [0.32]	4.31 [0.40]	3.31 [0.04]	U	U	(1.20)
	B11	3.99 [0.19]	U	3.20 [0.03]	U	U	U	(0.22)
	Mean	**3.85±0.47 [0.25± 0.35]**	**2.66±1.85 [0.10± 0.10]**	**3.86±0.51 [0.27± 0.35]**	**0.33±1.05 [0.0040± 0.01]**	**0.33±1.05 [0.0042± 0.01]**	**U**	**(0.74)**

(Table 4.5 continued)

Group	Chicken No.	Sampling day post challenge						(Total viral load in EID_{50}/ml)
		2	**3**	**4**	**7**	**14**	**21**	
C: Commercial H6N2 vaccine	C1	3.69 [0.10]	3.88 [0.15]	U	U	U	U	(0.24)
	C2	3.68 [0.09]	U	3.41 [0.05]	U	U	U	(0.14)
	C3	3.62 [0.08]	U	U	U	U	U	(0.08)
	C4	3.83 [0.13]	3.73 [0.10]	3.46 [0.06]	U	U	U	(0.29)
	C5	U	3.77 [0.11]	4.19 [0.30]	U	U	U	(0.42)
	C6	3.48 [0.06]	U	3.46 [0.06]	U	U	U	(0.12)
	C7	4.34 [0.42]	U	U	U	U	U	(0.42)
	C8	3.63 [0.08]	4.50 [0.61]	4.13 [0.26]	U	U	U	(0.96)
	C9	3.50 [0.06]	U	U	U	U	U	(0.06)
	C10	3.48 [0.06]	U	4.03 [0.21]	4.05 [0.22]	U	U	(0.49)
	C11	U	U	U	3.26 [0.04]	U	U	(0.04)
	C12	3.82 [0.13]	4.08 [0.23]	3.77 [0.12]	3.45 [0.06]	U	U	(0.53)
	Mean	**3.09±1.46 [0.10± 0.11]**	**1.66±2.06 [0.10± 0.18]**	**2.20±1.96 [0.09± 0.11]**	**0.90±1.63 [0.03± 0.06]**	**U**	**U**	**(3.79)**
D: Non-vaccinated control	D1	U	U	3.18 [0.03]	U	U	U	(0.03)
	D2	U	3.75 [0.11]	3.13 [0.03]	U	U	*	(0.14)
	D3	4.40 [0.48]	4.45 [0.55]	4.69 [0.94]	4.18 [0.30]	3.42 [0.05]	U	(2.32)
	D4	3.56 [0.07]	U	U	U	U	U	(0.07)
	D5	3.39 [0.05]	4.33 [0.42]	3.77 [0.11]	U	3.43 [0.05]	U	(0.63)
	D6	4.01 [0.20]	U	3.63 [0.08]	U	U	U	(0.28)
	D7	U	3.51 [0.06]	3.80 [0.12]	U	U	U	(0.19)
	D8	U	U	U	U	U	U	(U)
	D9	3.45 [0.05]	3.77 [0.11]	3.83 [0.13]	U	U	U	(0.30)
	D10	3.68 [0.09]	3.56 [0.07]	U	U	U	U	(0.16)
	D11	4.07 [0.23]	4.37 [0.46]	3.46 [0.06]	U	U	U	(0.74)
	D12	U	4.75 [1.08]	3.72 [0.10]	3.18 [0.03]	U	U	(1.22)
	Mean	**2.21±1.97 [0.10± 0.14]**	**2.71±2.03 [0.24± 0.33]**	**2.77±1.71 [0.13± 0.26]**	**0.61±1.45 [0.03± 0.09]**	**0.57±1.33 [0.01± 0.02]**	**U**	**(6.08)**

U-undetected/ below the limit of detection of 1000 viral copies

* chicken D2 was euthanized 16 days post challenge for humane reasons unrelated to viral challenge

Table 4.6. Mean group qRT-PCR results with standard deviation for cloacal swabs. EID_{50}/ml viral titers are indicated with the proportion of shedders in parenthesis.

Description	Group(s)	Sampling day post challenge						Total mean group titers (EID_{50}/ml)	Total individual titers (EID_{50}/ml)
		2	3	4	7	14	21		
Group EID_{50}/ml titers	A	0.04† (5/12)‡	0.01† (1/12)‡	0.02† (2/12)‡	0.01† (3/12)‡	U	U	0.07†	0.86¥
	B	0.25† (10/10)‡	0.10† (7/10)‡	0.27† (10/10)‡	0.0040† (1/10)‡	0.04† (1/10)‡	U	0.62†	0.74¥
	C	0.10† (10/12)‡	0.10† (5/12)‡	0.09† (7/12)‡	0.03† (3/12)‡	U	U	0.32†	3.79¥
	D	0.10† (7/12)‡	0.24† (8/12)‡	0.13† (9/12)‡	0.03† (2/12)‡	0.01† (2/12)‡	U	0.51†	6.08¥
Comparison between groups	A vs. C (C/A)	2.49 X§ (A:-59.86%)¶	19.38 X§ (A:-94.84%)¶	5.24 X§ (A:-80.93%)¶	3.01 X§ (A:-66.76%)¶	-	-	4.43 X§ (A: -77.44%)¶	4.43 X§ (A:-77.44%)¶
	A vs. D (D/A)	2.41 X§ (A:-58.55%)¶	45.84 X§ (A:-97.82%)¶	8.01 X§ (A -87.51%)¶	3.18 X§ (A:-68.54%)¶	-	-	7.11 X§ (A:-85.93%)¶	7.11 X§ (A:-85.93%)¶
	B vs. C (C/B)	0.41 X§ (B:+59.39%)¶	1.02 X§ (B:-2.37%)¶	0.33 X§ (B: +66.99%)¶	6.43 X§ (B:-84.45%)¶	-	-	0.51 X§ (B: +49.26%)¶	5.13 X§ (B:-80.52%)¶
	B vs. D (D/B)	0.39 X§ (B: +60.67%)¶	2.42 X§ (B:-58.73%)¶	0.50X§ (B: +49.59%)¶	6.80 X§ (B:-85.29%)¶	2.07 X§ (B:-51.58%)¶	1.67 X§ (B:-51.65%)¶	0.81 X§ (B: +18.61%)¶	8.23 X§ (B:-87.86%)¶
	C vs. D (D/C)	0.97 X§ (C:+3.16%)¶	2.37 X§ (B:-57.73%)¶	1.53 X§ (B:-34.52%)¶	1.06 X§ (B:-5.36%)¶	-	-	1.60 X§ (B:-37.66%)¶	1.60 X§ (B:-37.66%)¶

† Mean viral titer (EID_{50}/ml); ‡ Positive birds; § X: Fold difference in viral titers; ¶ Percentage reduction (-) or increase (+) in viral titers in comparison to the control group; ¥ Sum of the individual viral titers (EID_{50}/ml) in a treatment group.

4.4. Conclusions

This chapter described the efficacy testing of plant-produced sub-lineage I and II H6 VLP vaccines in SPF White Leghorn chickens, in comparison to the commercial whole inactivated H6N2 virus vaccine (AVIVAC® AI) and a non-vaccinated control group. The H6 VLP vaccines were formulated with a commercial mineral oil adjuvant and administered intramuscularly to facilitate comparison to the oil-emulsion commercial vaccine, which contains ≥ 10^8 EID_{50} per recommended dose (0.5 ml) according to the label. Since we did not perform a preliminary *in vivo* chicken trial to determine the minimal efficacy dose in chickens, a limitation of the study, we opted for a high antigenic mass dose (768 HAU/dose) for the sub-lineage I VLP vaccine and the maximum antigenic mass dose for the sub-lineage II VLP vaccine (48 HAU/dose). As per standard practice in the field, chickens received a booster vaccination four weeks after the primary immunization and were challenged two weeks later with a live H6N2 field virus (10^6 EID_{50}/0.06 ml).

HA- and NP-antibody specific antibody responses were determined four weeks after the primary immunization, two weeks after the booster immunization and two weeks after viral challenge. A single dose of the sub-lineage I VLP vaccine elicited an immune response (GMT of 9.3 log_2) comparable to two doses of the commercial vaccine (GMT of 8.8 log_2), as assessed against the respective closely related or homologous HI antigen, demonstrating the high potency of the H6 sub-lineage I VLP vaccine. For the sub-lineage II VLP vaccine the immune response was substantially lower after two doses (GMTs of 5.3 log_2 and 4.0 log_2 against the 2002 and 2016 HI antigens, respectively), most probably due to the low antigenic mass dose of the vaccine. Two weeks after viral challenge, HA-specific antibody titers against the challenge virus HI antigen (2016) were high (GMT > 7.9 log_2) for all of the groups. In addition, positive NP-specific antibody responses, as determined using NP ELISA assay, were only detected in 2/12 (A3 and A10) chickens in the sub-lineage I VLP vaccine, indicating the inhibition of viral replication in these birds. All 10 birds in the sub-lineage II VLP vaccine group, as well as the commercial vaccine and non-vaccinated groups, had positive NP titers two weeks after viral challenge.

For LPAI viruses, the prevention or reduction of viral shedding from the respiratory and gastrointestinal tracts is the main determinant of vaccine efficacy as clinical signs are not normally observed under experimental conditions. Overall, the total mean oropharyngeal shedding (EID_{50}/ml) for each group was 5.09- and 6.51-fold higher compared to cloacal viral titers in log_{10} terms. In comparison to the non-vaccinated control, vaccination with the sub-lineage I H6 VLP vaccine resulted in an overall reduction in oropharyngeal and cloacal

shedding (EID_{50}/ml) of 100.45–fold (99.00% reduction) and 7.11–fold (85.93% reduction), respectively, reduced the proportion of shedders throughout the assessment phase and shortened the duration of shedding by at least a week. In comparison to the non-vaccinated control, the sub-lineage II H6 VLP vaccine resulted in an overall reduction in oropharyngeal shedding (EID_{50}/ml) of 5.40-fold (84.47% reduction), but a slight increase of 18.61% (0.81-fold) in cloacal shedding titers. It is suspected that an increase in the antigenic mass dose of the sub-lineage II VLP vaccine could lead to a greater reduction in viral shedding titers and the proportion of shedders, as well as shorten the duration of shedding in vaccinated SPF chickens, as observed with the sub-lineage I VLP vaccine. In the commercial vaccine group the cost of antigenic mismatch between the vaccine and challenge viruses was evident as the vaccine not only failed to effectively reduce viral shedding in comparison to the non-vaccinated control group, but exacerbated oropharyngeal shedding until 21 days post challenge with a significant increase in mean group viral titers (6.96 log_{10} vRNA copies/ml) at 7 dpc. In comparison to the non-vaccinated control, the commercial vaccine resulted in an overall increase in oropharyngeal viral shedding (EID_{50}/ml) of 4.39% (0.96-fold) and a reduction in cloacal viral shedding of 1.6-fold (37.66%) with cloacal shedding ceasing at least a week before the non-vaccinated control group. It is suspected that the elevated oropharyngeal viral titers in comparison to the non-vaccinated control are due to vaccine-induce antigenic escape mutants, and follow-up investigations are in progress at the time of writing.

The capacity to differentiate between infected and vaccinated animals (DIVA) is of great importance in the field and is one of the characteristics of an ideal avian influenza vaccine (Swayne and Kapczynski, 2017). As such, NP-antibody responses were monitored using commercial ELISA kits to demonstrate the capacity of H6 VLP vaccines to differentiate between vaccinated chickens. Prior to viral challenge, NP ELISA results were consistently negative in the chickens vaccinated with the sub-lineage I and II VLP vaccinated, while strong NP-antibody responses were detected in all 12 chickens prime-boost vaccinated with the whole inactivated virus vaccines, as expected. Although HA is the primary target for neutralizing antibodies, immune responses are elicited against the full complement of viral proteins present in the avian influenza vaccine (prior to viral challenge) or the live replicating challenge virus (after viral challenge). The H6 VLPs contain only HA (and M2) protein, without any genetic material, which facilitates the differentiation between field infected and vaccinated chickens with a combination of appropriated serological tests. For example, the presence of HA- and NP-specific antibodies indicates exposure to a field virus, whereas the presence of HA-specific antibodies but absence of NP-specific antibodies indicates a vaccine

response in chickens. Alternatively, markers could be engineered into the VLP for DIVA (Roy and Stuart, 2013). In addition, plant-based VLP vaccines offer additional advantages over traditional inactivated vaccines: 1) strict bio-containment measures are not required as a live virus is not employed at any stage of the production; 2) SPF eggs are not required, which may have supply and animal ethics considerations; 3) they do not harbour human pathogens; and 4) as transient plant-based production is employed, the product is environmentally safe (D'Aoust *et al.,* 2010; Shoji *et al.,* 2011; Soema *et al.,* 2015; Moustafa *et al.,* 2016). Thus, plant-produced VLPs are DIVA compliant, ethical, bio-secure, sustainable and environmentally safe.

Due to the low manufacturing costs of plant-based expression, this platform is ideal for the production of veterinary vaccines, with the regulatory requirements for purity typically less stringent in comparison to human vaccines (Meeusen *et al.,* 2007). In this study, 40 g of infiltrated leaf material yielded sufficient H6 sub-lineage I VLPs for 400 vaccine doses, which means that, conservatively estimated, more than 5,000 chickens could be prime-boost vaccinated per kilogram (kg) leaf material, or given in view of the results after a single administration, 10,000 chickens could potentially be immunized with one kg of leaf material. Kilany *et al*. (2016) determined that inactivated H9N2 vaccines containing at least 250 HAU/dose elicited protective antibody titers and reduced virus shedding in SPF chickens. In this study, the antigenic dose of the H6 sub-lineage I VLP was 3-fold higher and up to 30,000 chickens could, therefore, be vaccinated from a kg of leaf material. For the sub-lineage II VLP vaccine the number of doses from 40 g of infiltrated leaf material was substantially less at an estimated 100 doses. However, the expression of sub-lineage II VLPs has since been improved to levels comparable to sub-lineage I VLPs (Chapter 3). The minimal efficacy of sub-lineage I and II VLP vaccines in chickens need to be established *in vivo*.

Chapter 5
General conclusion and future considerations

Vaccination of poultry against avian influenza is an effective disease control measure preventing the spread of this zoonotic disease to humans. Although the cost of a vaccine per bird is low, varying from $0.16 to $0.04 (WHO, 2012), the global poultry influenza vaccine market is massive. Between 2002 and 2010, 113.9 billion doses of licensed H5/H7 HPAI vaccines and 5.76 billion doses of licensed H5/H7 LPAI vaccines were administered to poultry, with the majority being oil-emulsified whole inactivated virus vaccines (Swayne *et al.,* 2011). Vaccination against avian influenza is aimed at eliciting an immune response (primarily hemagglutinin-specific antibodies) that protects against clinical signs of disease (morbidity and mortality), as well as replication and shedding of the field virus from the respiratory and gastrointestinal tracts (Swayne and Kapczynski, 2017). An ideal avian influenza vaccine needs to be cost-effective, applicable in multiple avian species, effective following a single vaccination, efficacious despite the presence of maternal antibodies, DIVA (differentiation between naturally infected and vaccinated animals) compliant, enable administration at 1 day of age or *in ovo*, have mass application potential, and very importantly, it needs to antigenically match the field strain for optimal protection (Swayne and Kapczynski, 2017). Although well established, effective and cost-effective, inactivated vaccines are typically administered subcutaneously or intramuscularly in prime-boost vaccination regimes after four weeks of age to compensate for the maternal antibody block, and DIVA entails the placing of sentinel birds with vaccinated flocks. In addition, egg-based production of avian influenza vaccines is dependent on specific-pathogen-free (SPF) embryonated chicken eggs (which may have supply and animal ethics considerations), require strict bio-containment measures as live viruses are involved, and can take up to six months (which can impede periodic updating of the vaccine to antigenically match the most recent field strain) (Gerdil, 2003; WHO, 2009; Soema *et al.,* 2015; Moustafa *et al.,* 2016). As antigenic differences between HA subtypes and between diverse strains within a specific HA subtype can significantly reduce the vaccine's effectiveness, it is recommended that vaccine strains should be re-evaluated at least every two to three years for efficacy against circulating field viruses and updated as needed (Swayne *et al.,* 2006; Swayne and Kapczynski, 2017; The World Organization for Animal Health (OIE), 2018).

The South African poultry industry has been beset by sporadic outbreaks of the low pathogenic avian influenza (LPAI) H6N2 subtype since the early 2000s, with two distinct lineages (sub-lineage I and II) identified from the onset. An inactivated oil-emulsion egg-

based whole inactivated virus vaccine derived from a 2002 H6N2 sub-lineage I field strain (AVIVAC® AI; Deltamune (Pty) Ltd., South Africa) was commercialized to protect flocks and is still in use under strictly regulated conditions. However, H6N2 field viruses have undergone substantial antigenic drift and antigenic diversity after more than a decade of vaccination, especially sub-lineage I (Rauff *et al.,* 2016; Abolnik *et al.,* 2019). Prior to the present study, the impact of these mutations on the efficacy (i.e. a significant reduction in viral shedding) of the commercial H6N2 vaccine against circulating field strains has not been determined in a clinical study, nor have any subsequent field isolates been developed as a replacement vaccine seed strain.

Plant-produced hemagglutinin (HA)-based influenza VLP vaccine candidates might be an attractive alternative to traditional egg-based vaccines. Globally, plant-produced hemagglutinin (HA)-based influenza VLPs for humans are in advanced clinical trials with proven efficacy, safety, speed of production, scalability and cost-effectiveness (D'Aoust *et al.,* 2008; D'Aoust *et al.,* 2010; Landry *et al.,* 2010). VLPs are recombinantly produced self-assembled protein structures that display conformational epitopes in a dense array on its surface without containing any core genetic material (Noad and Roy, 2003). As a result of its "virus-like" structure, VLPs are capable of eliciting potent antibody responses, as well as cellular immune responses through interaction with antigen presenting cells (Bright *et al.,* 2007; Quan *et al.,* 2007; Landry *et al.,* 2014). In addition, the lack of infectious genetic materials means that strict bio-containment measures are not required and that DIVA can be attained using a combination of appropriate serological tests (Liu *et al.,* 2013). The transient production of VLPs in plants offer additional advantages of cost-effective scalability and glycosylation of the target protein, which is required for immunogenicity and stability of VLP antigens (D'Aoust *et al.*, 2010; Kim *et al.,* 2014; Kolotilin *et al.,* 2014; Walwyn *et al.,* 2015; Nandi *et al.,* 2016). One of the greatest advantages of plant-produced VLPs, however, is the speed of production. Once the HA sequence of the latest influenza strain is available, a fully formulated influenza vaccine can be produced within four weeks, with up to 30 million doses of the VLP vaccine being produced within 3 months (D'Aoust *et al.,* 2008; D'Aoust *et al.,* 2010; Margolin *et al*., 2018). Given the short production time, transient plant-based production systems are considered be the best manufacturing platform to combat bioterrorism and pandemics and enables prompt updating of avian influenza vaccines to antigenically match the latest field strain for optimal efficacy (D'Aoust *et al.,* 2010; Morris *et al.,* 2017). The use of plant-produced influenza VLP vaccines have not been established for the poultry industry.

In this study, as a proof of concept, sub-lineage I and II HA-based H6 influenza VLPs were transiently produced in *Nicotiana benthamiana* plants to assess the suitability of this technology for poultry in South Africa. The first objective of the study was to develop an *Agrobacterium*-mediated transient plant-based production platform for the manufacturing of H6 influenza VLPs, using the plant expression vector pEAQ-HT. The following parameters were assessed to maximize the yield of H6 VLPs in *N. benthamiana*: 1) the codon optimization strategy (chicken, human or plant codon optimized versions of the H6 sub-lineage I HA gene), 2) the *A. tumefaciens* strain (AGL-1, GV3101::pMP90 or LBA4404), 3) the co-expression with influenza M2, 4) the addition of acetosyringone, a potent chemical inducer of *A. tumefaciens* virulence genes, and 5) the *A. tumefaciens* culture density (OD_{600} = 0.5, 1, 1.5 or 2). Collectively, the chicken codon optimized variant, *A. tumefaciens* strain AGL-1, co-expression with M2, the inclusion of acetosyringone in the infiltration buffer and a culture density of 1 to 1.5 were selected for subsequent transient expression of H6 avian influenza VLPs in *N. benthamiana*.

Appropriate downstream processing (DSP) is pivitol for the production of a commercially viable product. The extraction buffer is essential to ensure protein stability and consequently, a critical factor for the successful recovery of plant-produced VLPs. In this study, three buffers were compared to identify the most appropriate buffer for the extraction of H6 avian influenza VLPs from *N. benthamiana* plant leaf tissue, with the accumulation of target protein peaking at 6 days post infiltration. PBS buffer supplemented with sodium metabisulfite (pH 7.4) was found to be at least as effective if not slightly superior to Tris buffer ((Tris(hydroxymethyl)aminomethane), pH 8.0; Landry *et al.,* 2010) for the extraction of H6 influenza VLPs, whereas extraction in Bicine buffer (pH 8.4; Thuenemann *et al.,* 2013) did not result in intact VLPs. The use of PBS as extraction buffer from the onset will avoid a final buffer exchange dialysis step prior to administration of the vaccine antigen, thereby reducing process time and production costs.

Immobilized metal affinity chromatography (IMAC) purification of histidine (His)-tagged proteins is a cost-effective and relatively simple method appropriate for commercial-scale production and was, therefore, explored for the purification of plant-produced avian influenza VLPs (Hu *et al.,* 1999; Lichty *et al.,* 2005; Kuo and Chase, 2011; Pereira *et al.,* 2012; Lojewska *et al.,* 2016; Zhou *et al.,* 2018). In this study, the addition of a poly (6X) -histidine (His)-tag at the N-terminus but not the C-terminus resulted in the expression of H6 HA_0, while maintaining correct protein folding and activity of H6 VLPs. For both the sub-lineage I and II H6 HA_0 genes, the addition of a N-His-tag enhanced protein expression levels and

consequently the quantity of H6 VLPs in comparison to the respective untagged versions. The addition of the Kozak consensus sequence did not enhance the translational efficiency of sub-lineage I or II N-His-tagged H6 HA_0 genes as a slight decline in yield was observed. Increased yields of recombinant proteins (including VLPs) following the addition of a His-tag have been reported previously with *Escherichia coli* as expression systems (Svenson *et al.,* 2006; Park *et al.,* 2015; Manuel-Cabrera *et al.,* 2016), but to my knowledge, has not been described for plant-based expression systems. Purification of the N-His-tagged VLPs using IMAC with either Protino® Nickel (Ni)-TED (tris-(carboxymethyl)-ethylenediamine) or Ni-IDA (iminodiacetic acid) resulted in poor recovery of the target protein, possibly due to the His-tag being only partially exposed. The addition of a linker sequence (encoding for four glycine residues and one serine residue) between the His-tag and the H6 HA_0 sub-lineage I gene to improve the accessibility of the tag further enhanced expression levels in comparison to the His-tagged construct, but did not improve the purification efficiency of the target protein via IMAC. Nevertheless, the enhanced protein yield and potential benefit of IMAC for the cost-effective purification of His-tagged plant-produced H6 VLPs warrants future investigation.

As a preliminary investigation into the larger scale production of H6 VLPs, the N-His tagged sub-lineage I VLP construct was introduced into *N. benthamiana* using vacuum infiltration (OD_{600} = 0.4; 30 mbar, 4 minute holding time) and the recovered recombinant protein purified using commercially-scalable filtration technology (i.e. depth filtration followed by tangential flow filtration (TFF)). The yield N-His tagged H6 HA_0 was conservatively estimated at 158.81 mg per kg leaf material (accounting for 1.4% to 2.5% of the total soluble protein). This yield for N-His tagged H6 HA_0 is comparable to previous reports for transiently-produced VLPs involving the pEAQ-HT expression vector (i.e. 50 mg/kg and 200 mg/kg) (D'Aoust *et al.,* 2008; Thuenemann *et al.,* 2013), illustrating the potential of this expression platform at commercial scale.

For efficacy testing, partially-purified sub-lineage I and II H6 VLPs were emulsified with commercial adjuvant (Montanide™ ISA 71 VG) and used to vaccinate SPF chickens at six and ten weeks of age, with viral challenge occurring two weeks after the booster immunization. Evaluation of HA-specific antibody titers revealed that a single dose of the plant-produced H6 sub-lineage I VLP vaccine (768 HAU/300 µl) elicited an immune response (H6-specific antibodies; geometric mean titer (GMT) of 9.3 $\log_2$) comparable to two doses of the commercial vaccine (GMT of 8.8 $\log_2$), as assessed against the respective closely related/homologous hemagglutination inhibition (HI) test antigen, illustrating the high potency of the H6 sub-lineage I VLP vaccine. In contrast, the lower vaccine dose of the sub-lineage

II VLP vaccine (48 HAU/300 µl) as a result of reduced expression levels at the time of the efficacy study was evident in the immune responses elicited following prime-boost vaccination (GMT of 5.4 log_2 and 4 log_2 against the 2002 and 2016 HI test antigens, respectively). Assessment of viral shedding revealed that the plant-produced sub-lineage I H6 VLP vaccine led to an overall decrease in oropharyngeal and cloacal shedding of 100.45–fold and 7.11–fold, respectively, reduced the proportion of shedders throughout the assessment phase and shortened the duration of shedding by at least a week in comparison to the non-vaccinated control. The sub-lineage II H6 VLP vaccine resulted in an overall reduction in oropharyngeal shedding of 5.40-fold, but a slight increase of 18.61% (0.81-fold) in cloacal shedding titers were obtained in comparison to the non-vaccinated control. It is suspected that an increase in the antigenic mass dose of the sub-lineage II VLP vaccine could enhance its efficacy in SPF chickens upon viral challenge.

In addition to demonstrating the potential of plant-produced H6 VLPs to elicit a potent humoral immune response and reduce viral shedding, several other findings of this efficacy study are also of great importance. Firstly, the DIVA compliance of plant-produced H6 VLPs were demonstrated using the combination of HI and commercial nucleoprotein (NP)-ELISA tests. Secondly, the cost of antigenic mismatch between the commercial inactivated H6N2 vaccine and challenge viruses was evident: despite high HA-specific antibody titers prior to viral challenge, the vaccine not only failed to effectively reduce viral shedding in comparison to the non-vaccinated control group, but exacerbated oropharyngeal shedding until 21 days post challenge with an overall increase in oropharyngeal viral shedding of 4.39% (0.96-fold). Thirdly, the necessity of updating the standard HI test antigen (2002) to ensure accurate screening of flocks for the presence of H6 LPAI was demonstrated, as 6/12 chickens tested negative for H6 HA-specific antibodies two weeks after viral challenge. Fourthly, the total mean oropharyngeal shedding for each group was 5.09- and 6.51-fold higher compared to cloacal viral titers in log_{10} terms, demonstrating the necessity of collecting oropharyngeal swabs in the field for accurate identification of H6N2 viruses. Lastly, shedding of the challenge virus from the respiratory tract of non-vaccinated birds was detected in some birds until at least 21 dpc, indicating the possible length of viral shedding (and consequently transmission) following natural infection in the field.

Given the numerous advantages of plant-produced VLPs as vaccines, such a platform is ideal for the production of a cost-effective vaccine against H6 AI for chickens in South Africa. In the efficacy study, 40 grams of infiltrated leaf material yielded sufficient H6 sub-lineage I VLPs for 400 vaccine doses, which means that, conservatively estimated, more than 5,000

chickens could be prime-boost vaccinated per kg leaf material or, in view of the results after a single administration, 10,000 chickens could potentially be immunized with one kg of leaf material. A limitation of this study, however, is that the minimal efficacy dose for the respective H6 VLP vaccines were not established. Kilany *et al*. (2016) determined that inactivated H9N2 vaccines containing at least 250 HAU/dose elicited protective antibody titers and reduced virus shedding in SPF chickens. In this study, the antigenic dose of the H6 sub-lineage I VLP was 3-fold higher and as up to 30,000 chickens could, therefore, possibly be vaccinated with a single immunization from one kg of leaf material. For the sub-lineage II VLP vaccine the number of doses from 40 grams of infiltrated leaf material was substantially less at an estimated 100 doses. However, the expression of sub-lineage II VLPs has since been improved to levels comparable to sub-lineage I VLPs and it is suspected that the number of vaccine doses for the sub-lineage II vaccine obtained from one kg of leaf material would be similar to that obtained with the sub-lineage I VLP vaccine in this study. However, the minimal efficacy of sub-lineage I and II VLP vaccines in chickens remains to be established *in vivo*.

A limitation of this production system, however, is the fact that a good manufacturing practice (GMP) facility for the production of plant-based vaccines and products is not yet a reality in South Africa. However, the Department of Science and Technology has invested in developing plant-made antibodies, vaccines and biologics during the last decade as part of the Bio-Economy strategy and the Tshwane Animal Health Innovation of the Technology Innovation Agency has invested more than 8 million USD in developing animal vaccines and related products, a number of which are plant-based (Morris *et al.,* 2017). The establishment of a validated portfolio of plant-based products sprouting from these and related research projects, and the knowledge obtained through commercialization of these products, could assist in the establishment of a GMP (which is required for veterinary vaccine products) or a current GMP (cGMP) facility in South Africa and pave the way for future plant-based products in terms of hurdles to overcome relating to regulation and legislation (Morris *et al.,* 2017). In addition, several GMP-compliant biopharming facilities have been established globally and a number of plant-based products have been approved by the Food and Drug Association (FDA) or are in advanced clinical trials, paving the way for plant-produced poultry vaccines (Chen and Lai, 2013; Takeyama *et al.,* 2015). Consequently, any insurmountable regulatory hurdles to overcome in commercialization of plant-produced VLP vaccines for poultry health are not foreseen.

Finally, the mass application potential of plant-produced H6 influenza VLPs could be investigated in future. In the United States of America, automated *in ovo* application is standard practice for vaccines against Marek's disease virus and infectious bursal disease for broiler chickens with benefits including earlier immunity, accurate and uniform dosage, reduced labour costs, no stress of chickens from handling, and reduced contamination (Negash *et al.,* 2004; Williams and Zedek 2010). Recently, Schädler *et al.* (2019) reported the *in ovo* application of laryngotracheitis VLPs produced in cell cultures had no visible adverse effects on the development and wellbeing of the embryos or hatched chickens, and resulted in an antibody-based immune response. Alternatively, the application potential of plant-produced H6 influenza VLPs in drinking water or as an edible vaccine, for example, could be investigated. The mass-application of plant-produced VLP vaccines to poultry would be another advantage of this production platform and a step closer in attaining an "ideal" avian influenza vaccine. In addition, the formulation for long-term storage could also be investigated. For example, Peabody *et al.* (2017) reported that spray drying of *Escherichia coli (E. coli)*-produced VLPs into a dry powder increased the long-term stability at room temperature and at 37^{o}C without affecting its immunogenicity, which overcomes the expense of cold-chain storage and transportation, as well as concerns of the product being accidentally exposed to either freezing or elevated temperatures. Overall, plant-produced VLPs hold enormous potential for the poultry industry for the control of avian influenza, as well as other diseases caused by viruses with a high mutation rate requiring the vaccines to be updated frequently. The utilization of this valuable platform for the development of efficacious, safe and DIVA compliant vaccines for poultry health is envisaged.

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Reference: 12/11/1/1/8

Professor Celia Abolnik
Department of Production Animal Studies
Faculty of Veterinary Science
University of Pretoria

Email: celia.abolnik@up.ac.za

Dear Prof. Abolnik,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your application, submitted on 02 November 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The entire study must be performed in the DAFF approved BSL3 facility (with DAFF compliance certificate number DAFF-CO2);
4. The study may only start after Ethics approval was obtained;
5. Serum samples may be stored at the Poultry Research BSL3 Facility;

6. The stored serum samples may only be used for further research after having obtained new Section 20 approval;
7. The stored serum samples may not be outsourced without prior written approval from DAFF;
8. This section 20 expires on the 26 of January 2019.

Title of research/study: "Evaluation of viral shedding and humoral immune responses of plant-produced H6 VLP vaccines against challenge with life H6N2 LPAI virus in a prime-boost regime."
Researcher (s): Tanja Smith
Institution: CSIR, Biosciences, Meiring Naude Rd, Pretoria
Your Ref./ Project Number:
Our ref Number: 12/11/1/18

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -11- 15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**The development of a plant produced virus-like particle vaccine against H6 avian influenza for chickens in South Africa**
PROJECT NUMBER	**V075-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**T Smith**

STUDENT NUMBER (where applicable)	**U_17398518**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Chickens *(Gallus gallus)***	
NUMBER OF SAMPLES	**60**	
Approval period to use animals for research/testing purposes		**July 2017- July 2018**
SUPERVISOR	**Prof. C Abolnik**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 15 August 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S*4285-15*

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**The development of a plant produced virus-like particle vaccine against H6 avian influenza for chickens in South Africa**
PROJECT NUMBER	**V075-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**T Smith**

STUDENT NUMBER (where applicable)	**U_17398518**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Chickens *(Gallus gallus)***	
NUMBER OF SAMPLES	**60**	
Approval period to use animals for research/testing purposes		**January 2018 – January 2019**
SUPERVISOR	**Prof. C Abolnik**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 16 January 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

CSIR Research Ethics Committee
PO Box 395 Pretoria 0001 South Africa
Tel: +27 12 841 4060
Fax: +27 12 841 2476
Email:R&DEthics@csir.co.za

19 March 2018

Dear: **Ms Tanja Smith**

Approval of Protocol: **The development of a plant produced virus-like particle vaccine against H6 avian influenza for chickens in South Africa.**

This is to confirm that your Protocol reviewed by the CSIR REC has been approved. The reference number of this research project is **Ref: 230/2017.**

This approval is granted under the condition that:

1. The researcher remains within the procedures and protocols indicated in the proposal, as well as the additions made to the procedures and protocols as indicated in the responses submitted to the questions of the REC, particularly in terms of any undertakings made and guarantees given.
2. The researcher notes that **any deviations to the approved project/protocol must be submitted to the REC for approval before implementation**.
3. The researcher remains within the parameters of any applicable national legislation, institutional guidelines and scientific standards relevant to the specific field of research.
4. This approval is **valid for one calendar year from the date of this letter**.
5. The researcher submit bi-annual progress reports to the REC
6. The researcher immediately alert the REC of any adverse events that have occurred during the course of the study, as well as the actions that were taken to immediately respond to these events.
7. The researcher alert the REC of any new or unexpected ethical issues that emerged during the course of the study, and how these ethical issues were addressed. If unsure how to respond to these unexpected or new ethical issues as they emerge, the researcher should immediately consult with the REC for advice.
8. The researcher submit a short report to the REC on completion of the research in which it is indicated (i) that the research has been completed; (ii) if any new or unexpected ethical issues emerged during the course of the study; and if so, (iii) how these ethical issues were addressed.

We wish you all of the best with your research project.

Kind regards

Prof Shenuka Singh

(CSIR REC Chair)

Ms Brenda Mapunya

(CSIR REC Secretariat)

Appendix B

Protein sequences used in the design of the synthetic H6 hemagglutinin (HA) and matrix protein 2 (M2) genes.

> A/chicken/South Africa/N2826/2016 (H6N2) (sub-lineage I) HA sequence (GenBank: MH170289)

MIAIIAIALLVSTGKSDKICIGYHANNSTTQVDTILEKNITVTHSIELLETQKEERFCRVLNKAPLDL
RECTIEGWMLGNPRCDILLEDQRWSYIVERPSASNGICYPGPLNEIEELRSLIGSGERVERFEMFPKS
TWNGVDTENGITRACSSSTGGSSFYRNLLWIIKNKSASYPVIKGTYNNTGNQPIIYFWGVHHPPDADR
QNNLYGSGDRYIRMGTESMHFAKGPEIAARPSVNGQRGRIDYYWSVLNPGETLNIESNGNFIAPRYAY
RFFSTNKKGVIFKSNLPIENCDAQCQTTLGVLRTNKTFQNVSPQWTGECPKYVKSKSLRLATGLRNVP
QVETRGIFGAIAGFIEGGWTGMIDGWYGYHHENSQGSGYAADRDSTQKAIDGITNKVNTIIDKMNTQF
EAVGHEFSNLERRIDNLNKRMEDGLLDVWTYNAELLVLLENERTLDLHDANVKNLYERVKSQLRDNAN
DLGNGCFEFWHKCDNDCMESVKNGTYDYPKYQDESKLNRQKIESVKLDNLGVYQILAIYSTVSSSLVL
VGLIIAMGLWMCSNGSMQCRVCI

> A/chicken/South Africa/BKR4/2012(H6N2) (sub-lineage II) HA sequence (GenBank: KX595260.1)

MIAIIVIAILASAGKSDKICIGYHANNSTTQVDTILEKNVTVTHSIELLETQKEERFCKILNKAPLDL
GECTIEGWILGNPQCDLLLGDQSWSYIVERPTARNGICYPGVLNEVEELKALIGSGEKVERFEMFPRN
TWRGVDTNSGVTKACPSSTGGSSFYRNLLWIIKSKSAAYPVIKGTYNNTGNQPILYFWGVHHPPDTNE
QNTLYGSGDRYVRMGTESMNFAKGPEIAARPAVNGQRGRIDYYWSVLKPGETLNVESNGNLIAPWYAY
KFVSTSNKGAVFKSNLPVEDCHAICQTAAGVLRVNKRFQNVSPLWIGECPKYVKSKSLRLATGPRNVP
QIETRGLFGAIAGFIEGGWTGLIDGWYGYHHENSQGSGYAADRESTQKAVDGITNKVNAIVDKMNTQF
EAVDHEFSNLERRIGNLNKRMEDGFLDVWTYNAELLVLLENERTLDLHDANVKNLFEKVKSQLRDNAN
DLGNGCFEFWHKCDNDCMESVKNGTYDYPNHQEESKLNRQEIESVKLENLGVYQILAIYSTVSSSLVL
VGLIIAIGLWMCSNGSMQCRICI

> A/New Caledonia/20/1999 (H1N1) M2 sequence (GenBank: HQ008884.1)

MSLLTEVETPIRNEWGCRCNDSSDPLVVAASIIGIVHLILWIIDRLFSKSIYRIFKHGLKRGPSTEGV
PESMREEYREEQQNAVDADDGHFVSIELE

Appendix C

A

DKICIGYHANNSTTQVDTILEKNITVTHSIELLETQKEERFCRVLNKAPLDLR**ECTIEGWMLGNPRCD**
ILLEDQRWSYIVERPSASNGICYPGPLNEIEELR**SLIGSGERVERFEMFPKSTWNGVDTENGITR**ACS
SSTGGSSFYR**NLLWIIK**NK**SASYPVIK**GTYNNTGNQPIIYFWGVHHPPDADR**QNNLYGSGDR**YIR**MGT**
ESMHFAKGPEIAARPSVNGQRGRIDYYWSVLNPGETLNIESNGNFIAPRYAYR**FFSTNKK**GVIFKSNL
PIENCDAQCQTTLGVLRTNK**TFQNVSPQWTGECPK**YVKSKSLRLATGLR**NVPQVETR**GIFGAIAGFIE
GGWTGMIDGWYGYHHENSQGSGYAADR**DSTQKAIDGITNKVNTIIDKMNTQFEAVGHEFSNLER**R**IDN**
LNKRMEDGLLDVWTYNAELLVLLENER**TLDLHDANVKNLYER**VKSQLR**DNANDLGNGCFEFWHK**CDND
CMESVK**NGTYDYPK**YQDESKLNR**QKIESVK**LDNLGVYQILAIYSTVSSSLVLVGLIIAMGLWMCSNGS
MQCRVCI

B

DKICIGYHANNSTTQVDTILEKNVTVTHSIELLETQKEERFCKILNKAPLDLGECTIEGWILGNPQCD
LLLGDQSWSYIVERPTAR**NGICYPGVLNEVEELK**ALIGSGEK**VERFEMFPR**N**TWR**G**VDTNSGVTKACP**
SSTGGSSFYRNLLWIIKSK**SAAYPVIK**GTYNNTGNQPILYFWGVHHPPDTNEQNTLYGSGDRYVR**MGT**
ESMNFAKGPEIAARPAVNGQRGRIDYYWSVLKPGETLNVESNGNLIAPWYAYK**FVSTSNKGAVFK**SNL
PVEDCHAICQTAAGVLRVNKR**FQNVSPLWIGECPK**YVKSK**SLRLATGPR**N**VPQIETR**GLFGAIAGFI
EGGWTGLIDGWYGYHHENSQGSGYAADR**ESTQKAVDGITNKVNAIVDKMNTQFEAVDHEFSNLERRIG**
NLNKRMEDGFLDVWTYNAELLVLLENER**TLDLHDANVKNLFEK**VKSQLR**DNANDLGNGCFEFWHK**CDN
DCMESVK**NGTYDYPNHQEESKLNRQEIESVK**LENLGVYQILAIYSTVSSSLVLVGLIIAIGLWMCSNG
SMQCRICI

C

MSLLTEVETPIRNEWGCRCNDSSDPLVVAASIIGIVHLILWIIDR**LFSKSIYRIFKHGLKRGPSTEGV**
PESMREEYREEQQNAVDADDGHFVSIELE

LC-MS/MS-based peptide sequence analysis for SDS-PAGE bands of approximately 62 kDa and 14 kDa, respectively. For the sub-lineage I (A) 62 kDa band, peptide sequences are mapped against the hemagglutinin protein sequence of A/chicken/South Africa/N2826/2016 (H6N2) for reference. For the sub-lineage II (B) 62 kDa band, peptide sequences are mapped against the hemagglutinin protein sequence of A/chicken/South Africa/BKR4/2012(H6N2) for reference. For the 14 kDa band, peptide sequences are mapped against the M2 ion channel protein sequence of A/New Caledonia/20/1999 (H1N1) for reference. Peptides identified with confidence (99%) are highlighted in green, with medium confidence are highlighted in yellow, and low confidence (0.1%) are highlighted in red. No peptides were identified for the non-highlighted grey regions of the protein sequences.