Plant-based vaccines for poultry viral diseases:

Avian influenza

By

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Abstract

Avian influenza virus (AIV) is a significant reason for mortality in poultry, causing substantial economic loss. The importance of AIV is increased due to the ability of several subtypes to cause zoonotic infection, with, in the case of H5N1, mortality rates approaching 60%. Haemagglutinin (HA) is the main antigen of AIV, and protecting antibodies are elicited against it in the host after viral infection. Control of viral diseases in poultry depends mainly on vaccination and high biosecurity levels.

Commercial vaccines are available for chickens' most common viral infections; however, viruses are still causing high economic losses. Vaccines that can be made at a cheap cost, are stable, and can be adapted to local virus strains are urgently needed. Such benefits are provided by plant-based vaccinations, which are appropriate for use in developing countries. The most predominant AIV strains in developing countries, especially Egypt, are H9N2 and H5N8. Virus-like particles (VLPs) have the same structure as the virus without inner nucleic acid. VLPs vaccines are safe and can stimulate both cellular and humoral immune responses in the host.

This project used *Agrobacterium tumefaciens* to introduce a plasmid encoding the codon optimised (HA) of H9N2 and H5N8 AIV into *Nicotiana benthamiana* leaves. Recombinant proteins and VLPs were transiently expressed and purified. The $H2N3_{HA}$ was transiently expressed in plants

without codon optimisation of the gene but at lower levels, which suggested that codon optimisation is important for enhancing the gene expression levels. VLPs for both H5 and H9 were identified by western blot and transmission electron microscopy (TEM). Each VLP preparation was formulated with an adjuvant for immunisation trials in mice and chickens and HA-specific antibodies were detected by enzyme-linked immunosorbent assay (ELISA).

Furthermore, sera were tested for their capacity to effectively neutralise heterologous pseudotype viruses presenting HA from heterologous strains in the H5 and H9 subtypes. In addition, cellular immune responses in mice were estimated by enzyme-linked immunosorbent spot (ELISpot). Mice were immunised with three H9 and H5 VLPs doses and elicited excellent haemagglutinin-specific antibodies, completely neutralising the heterologous pseudotypes. Additionally, a high number of interferon-γ secreting splenocytes were detected on restimulation of cells from immunised mice with H9 and H5 VLPs through the ELISpot technique. Chickens were immunised with one dose of H5 VLPs eliciting haemagglutinin-specific antibodies, partially neutralizing the heterologous H5 pseudotypes. Plant-based VLP vaccines are an excellent alternative to the traditional vaccine with significant advantages for producing a vaccine candidate quickly with high potential for the poultry industry.

Declaration

The author, Ola Elbohy, has obtained the relevant research ethical approval for the research described in this work. The author declares that she has followed the ethical standards required in terms of the Code of Ethics of the University of Nottingham and the research policy guidelines.

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AGE	agarose gel electrophoresis
AIV(s)	avian influenza virus(es)
APCs	antigen-presenting cells
ATP	adenine triphosphate
BSA	bovine serum albumin
BTNL	butyrophilin-like
CD	cluster differentiation
CF	complement fixation
CPE	cytopathic effects
cRNA	complementary ribonucleic acid
CT	cytoplasmic tail
CTL	cytotoxic T-lymphocyte
CVMP	Committee for Veterinary Medical Products
DARPA	Defense Advanced Research Projects Agency
DCs	dendritic cells
Δenv	delta envelope
DIVA	differentiating infected from vaccinated animals
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunosorbent spot
ER	endoplasmic reticulum
ERKs	extracellular signal-regulated kinases
FA	Freund’s adjuvant
FDA	Food and Drug Administration
GCN4pII-IgMFc	C-terminal isoleucine zipper chicken immunoglobulin
GMP	good manufacturing practice
HA	haemagglutinin
HEK	human embryonic kidney
HI	haemagglutination inhibition
HIV	human immunodeficiency virus core
HRP	horseradish peroxidase
HPAI(V)	highly pathogenic avian influenza (virus)
IAV(s)	influenza A virus(es)

IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-2	interleukin 2
IFN-γ	interferon-gamma
ILTV	infectious laryngotracheitis virus
IMAC	immobilized metal affinity chromatography
iNOS	inducible nitric oxide synthase
LB-Broth	Luria Bertani Broth
LC3	microtubule-associated protein 1A/1B–light chain
LPAI(V)	low pathogenicity avian influenza (virus)
LSD	lesion simulating disease
M2	matrix protein 2
MAVS	mitochondrial antiviral signalling protein
MDA5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
NA	neuraminidase
NaCl	sodium chloride
NDS	new drug submission
NEP	nuclear export protein
NK	natural killer
NLG	N-linked glycosylation
NLS	nuclear localization signals
NO	nitric oxide
NP	nucleoprotein
NS1	non–structural protein 1
OD	optical density
O/W	oil in water
P	polymerase
PA	polymerase acidic
PB	polymerase basic
PBS	phosphate buffer saline

PCR	polymerase chain reaction
pHACS	poly basic haemagglutinin cleavage site
Pol-II	polymerase II
PVNT	pseudotyped virus neutralisation test
PRRs	pattern recognition receptors
PR1a	Pathogenesis related protein-1a
QVLP	quadrivalent virus-like particle
RBCs	red blood cells
RBD	receptor binding domain
rFP-AIV-H5	recombinant fowlpox avian influenza-H5
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT– qPCR	reverse transcriptase quantitative polymerase chain reaction
SA	sialic acid
SAP04	Specified Animal Pathogens Order
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOC	Super Optimal broth with Catabolite repression
SUMO	Small ubiquitin-related modifier
$TCID_{50}$	tissue culture infective $dose_{50}$
Th	T helper cells
TLR	toll-like receptor
TMD	transmembrane domain
TNF-α	tumour necrosis factor-alpha
VE	vestigial esterase
VSV-G	vesicular stomatitis virus glycoprotein G
VLP	virus-like particle
W/O	water in oil
%	Percentage
˚C	Degrees Celsius

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1.1. Avian flu

“Avian flu” is a contagious viral disease caused by the influenza A virus (IAV), affecting many species. Influenza A viruses occur worldwide, and human epidemics and pandemics cause high morbidity and mortality. Human influenza epidemics occur yearly during winter and autumn in temperate areas and cause a high mortality and morbidity rate yearly (Shrestha et al., 2015). In humans, it has been recorded that yearly epidemics are estimated to cause about 3–5 million infections and 290,000 to 650,000 respiratory deaths annually (WHO, 2016). Historically, the most crucial human influenza pandemic was the 'Spanish flu,' which occurred in 1918, with high fatalities worldwide (Johnson and Mueller, 2002). Other critical human pandemics were in 1957 (the Asian flu) and 1968 (the Hong Kong flu), but they were less serious than the Spanish flu (Horimoto and Kawaoka, 2001, Horimoto and Kawaoka, 2005).

The first documentation of the avian influenza virus (AIV) was made in Italy in 1878 when scientists distinguished a poultry disease from other infections with elevated mortality rates. The disease, known as fowl plague, was primarily mistaken for the acute septicaemic phase of fowl cholera (an infection caused by *Pasteurella multocida)*. In 1880, scientists documented that the disease differed from fowl cholera and called it *Typhus exudatious gallinarum*. Scientists recorded that a filterable virus caused the fowl plague

in 1901. The fowl plague was documented as an IAV by identifying IAV-type-specific ribonucleoprotein in 1955 (Lupiani and Reddy, 2009). The first documentation of IAV in wild ducks was in 1972, and since that time, many studies have revealed that wild birds are reservoir hosts for IAV (Slemons et al., 1974).

1.2. Types of influenza virus

The Orthomyxoviridae family contains seven species, including the influenza viruses (A, B, C, and D), Thogotovirus, Isavirus, and Quaranjavirus. The initial four genera include viruses that lead to influenza in vertebrates; isaviruses affect salmon. Thogotoviruses are arboviruses causing disease in vertebrates and invertebrates, including mosquitoes and ticks (ICTV, 2019). In 1931, IAV was initially isolated from pigs and then from humans in 1933 (Shope, 1931, Smith et al., 1933). IAV causes severe and potentially fatal diseases (Cox et al., 2004). The natural reservoirs of most IAV are wild birds; they also infect poultry, pigs, horses, and occasionally sea mammals (Webster et al., 1992).

The major differences between the four significant influenza types (A, B, C, and D) are summarised in Table 1.1.

Table 1.1 Comparison of the main properties of influenza viruses, adapted from (Racaniello and Palese, 1979, Hatta and Kawaoka, 2003, Asha and Kumar, 2019, Sederdahl and Williams, 2020).

	Influenza A	**Influenza B**	**Influenza C**	**Influenza D**
Species affected	Wild aquatic birds, poultry, horses, pigs, dogs, and occasionally sea mammals and bats	Humans and seals	Humans, Pigs, cattle, and dogs	Cattle and pigs
Number of gene segments	8	8	7	7
Surface glycoprotein	HA and NA	HA and NA	HEF (Haemagglutinin–Esterase–Fusion)	HEF (Haemagglutinin–Esterase–Fusion)
HA and NA subtypes	18 HA and 11 NA	None	None	None

Number of major proteins	10	11	9	9
Epidemiological feature	Causes epidemics and pandemics	Less severe outbreaks and no pandemic	No epidemics or pandemics	No epidemics or pandemics

1.3. Influenza A viruses

1.3.1. Morphology

IAV is spherical or filamentous; spherical virions are 100 nm in diameter, and filamentous virions are about 300 nm in length. The influenza A virion is covered with haemagglutinin (HA) glycoprotein spikes, which are rod-like and constitute 80% of the surface proteins. Neuraminidase (NA), which is mushroom-shaped, constitutes 17% of the surface proteins, with minor components of M2 (matrix protein 2) represented by a few molecules bulging from the envelope. The envelope and its three integral membrane proteins cover an M1 matrix protein surrounding the virion core (Figure 1.1). The nuclear export protein (NEP) is covered by the M1 matrix protein. The core consists of a ribonucleoprotein (RNP) complex, which is comprised of the viral RNA segments covered with nucleoprotein (NP) and the polymerase complex, composed of two basic and one acidic polymerase subunits (PB1, PB2, and PA) (Palese, 2007).

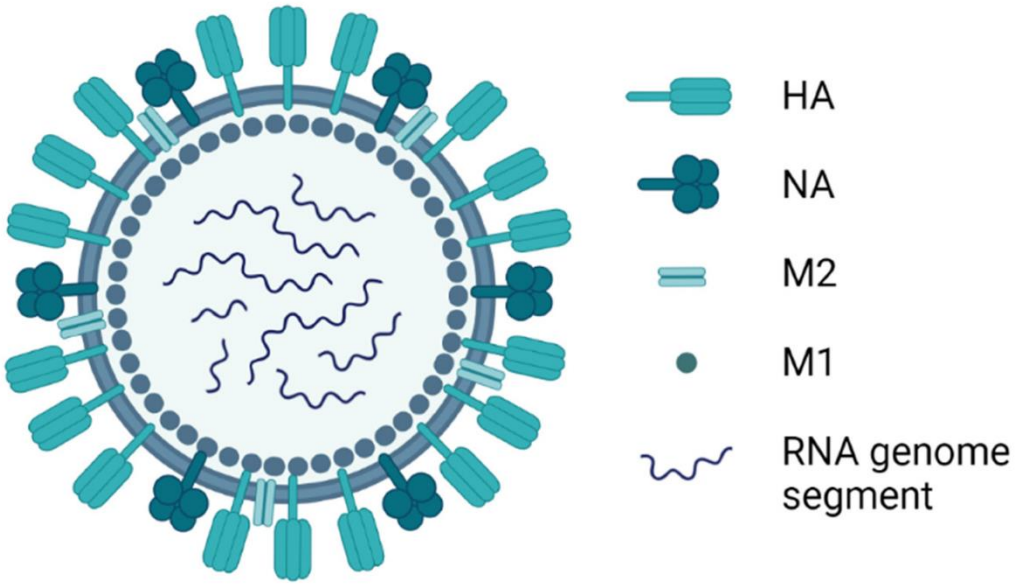


Figure 1.1. Structure of the influenza A virus. Figure adapted from Viralzone (2010) and created with BioRender.com.

1.3.2. Genome

The IAV genome comprises eight single-stranded, negative-sense viral RNA (vRNA) segments. The eight segments of IAV are numbered in order with decreasing length (Table 1.2). Influenza A virions contain three discrete polymerases (P) proteins (Lamb, 1974, Lamb and Choppin, 1976). The three P proteins are linked with transcriptionally active ribonucleoprotein structures and about 30–40 molecules of each P protein per virion particle (Inglis et al., 1976). Two of the P polypeptides are basic, and one is acidic (Horisberger, 1980). Using protein gel electrophoresis, the more quickly migrating basic P protein encoded by RNA segment one was called PB2, the slowly migrating basic P protein encoded by RNA segment two was named PB1, and the acidic P protein encoded by RNA segment three was

named PA (Horisberger, 1980, Ulmanen et al., 1981, Winter and Fields, 1982).

PB2 identifies and binds to a structure at the host-cell RNA 5' end to prime influenza virus transcription (Nichol et al., 1981, Ulmanen et al., 1981, Braam et al., 1983). ANP32A is a protein present in chickens that assists the function of the AIV polymerases complex in human cells. For this function, the AIV polymerase cannot effectively use shorter ANP32 proteins, such as mammalian ANP32A or ANP32B. The acquisition of certain mutations, such as PB2 (E627K), facilitates polymerase function enhanced by the shorter ANP32 proteins in mammalian hosts. PB2 adaptation is necessary to facilitate the human pandemic possibility of AIVs. Interrupting the interaction of the virus with ANP32A might be a unique method for virus control (Long et al., 2016).

Influenza virus encodes the PB1 on segment 2; in some strains of IAVs, the segment additionally encodes the protein PB1-F2, which has a pro-apoptotic function (Chen et al., 2001). Another viral protein (N40) translated from segment two has been detected in some IAV isolates (Chen et al., 2001, Wise et al., 2009). PB1 is mainly responsible for the chain initiation and elongation steps of attaching the first base to the RNA primer derived from the host (Braam et al., 1983).

The influenza virus encodes the PA on segment three, which is essential for the transcription and replication of the viral genome. The segment also encodes the protein PA-X, which controls host response and viral virulence through endonuclease activity on host ssRNA (Maier et al., 2008, Jagger et al., 2012, Bavagnoli et al., 2015).

Segment 4 of IAV encodes HA, the virus glycoprotein spike that binds to the sialic acid (SA) on the cell membrane, establishing the attachment of the virus to the host cell membrane. Following endocytosis, HA starts the infection process by mediating the fusion of the endocytosed virus particle with the endosomal membrane, releasing the viral cores into the cytoplasm. Proteolytic cleavage of the HA of new virus particles is required to render viruses infectious by cleavage of HA0 to HA1 and HA2, which remain covalently associated (Figure 1.2). The HA is the main virus antigen responsible for eliciting neutralizing antibodies in the host, and recurring influenza epidemics are mostly related to changes in its antigenic structure (Wilson et al., 1981).

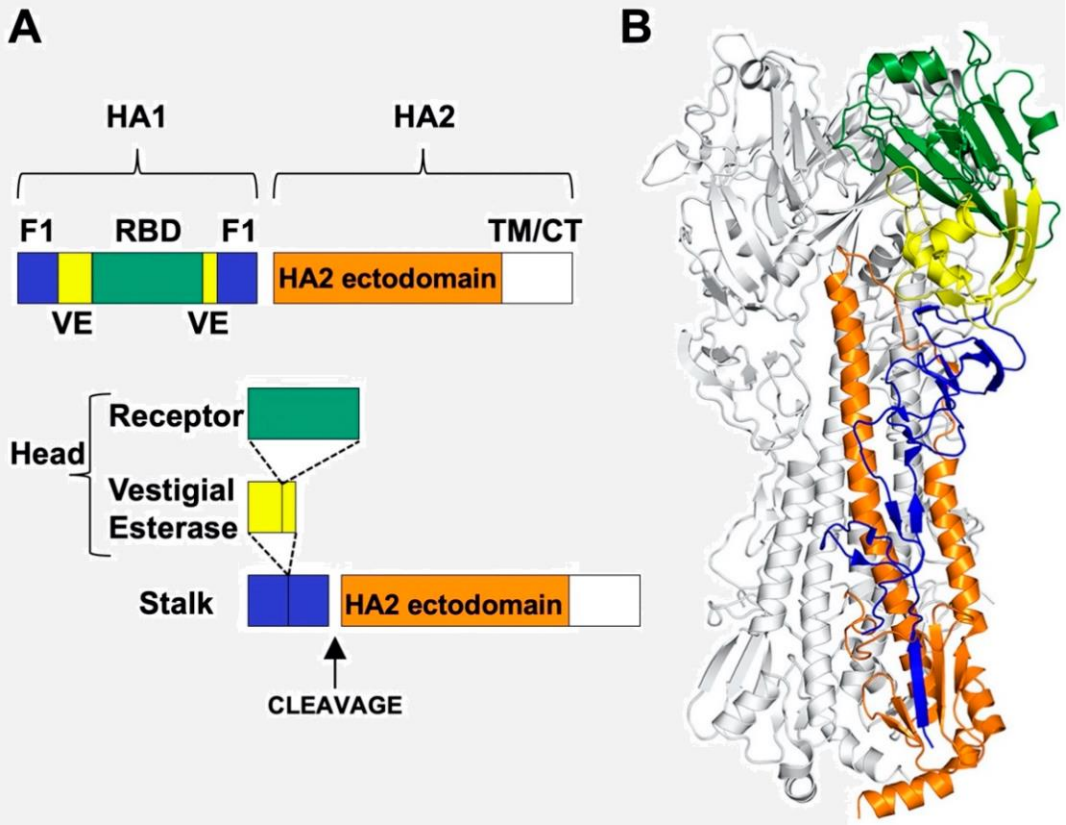


Figure 1.2. Structure of the HA domains. Figure A shows the structure of the HA1, which includes F: fusion domain, VE: vestigial esterase domain, and RBD: receptor binding domain, while HA2 includes the HA2 ectodomain, TM: transmembrane domain, and CT: cytoplasmic tail. Figure B shows the 3D structure of the HA domains. The figure was modified from (Russell, 2021).

Segment 5 of IAV encodes the NP, the main structural protein interacting with the viral RNA segment to form the RNP. Accompanying the RNP, there are three transcriptase-associated polypeptides: PB1, PB2, and PA (Choppin and Compans, 1975).

Segment 6 of the IAV encodes the NA protein, a main spike glycoprotein of the virion, and is significant both for its biological activity in removing SA from glycoproteins and as a primary antigenic determinant that undergoes antigenic variation (Colman and Ward, 1985, Hatta and Kawaoka, 2003).

IAVs can be classified into several subtypes according to the surface antigens (HA and NA). There are 18 subtypes of HA and 11 subtypes of NA, which are expressed as H1–H18 and N1–N11 (Webster and Govorkova, 2014). Of those subtypes, the first 16 HA and 9 NA were detected in waterfowl, and the last two HA and NA were recorded in bats (Tong et al., 2012, Tong et al., 2013).

Three RNA transcripts have been recognised that are derived from segment 7: a transcript encoding the M1 protein, which is a highly abundant polypeptide in the virion, a spliced messenger ribonucleic acid (mRNA) encoding the M2 protein that is expressed at the cell surface, and an alternatively spliced (Wise et al., 2012).

Finally, segment 8 encodes NS1 and NEP. NS1 significantly inhibits host immune responses by decreasing interferon (IFN) response (Hale et al., 2008). NEP plays a vital role in exporting RNPs from the nucleus to the cytoplasm during viral replication and regulation of virus transcription and replication processes (Robb et al., 2009).

Table 1.2. Illustration of the IAV gene segments and functions modified from Bouvier and Palese (2008).

Segment	Encoded protein(s)	Protein length in amino acids	Protein function
1	PB2	759	mRNA cap recognition
2	PB1	757	RNA elongation during replication
	PB1-F2	87	Apoptosis
	N40	718	Interaction with polymerase complex
3	PA	716	Endonuclease activity
	PA-X	252	It affects the host response and triggers viral virulence
4	HA	550	Main virus antigen, receptor binding, and responsible for generating neutralising antibodies in the host
5	NP	498	Regulation of the nuclear import
6	NA	454	Sialic acid activity, and virus release from cells
7	M1	252	Viral assembly and budding
	M2	97	Virus uncoating and assembly
8	NS1	230	Interferon antagonist protein

NEP	121	Responsible for the export of RNA from the nucleus

1.3.3. Infection cycle of influenza A viruses

1.3.3.1. Virus attachment and entry

IAV starts the infection process via the HA spike. The HA spike attaches the virus to the host membrane with terminal SA residues (Weis et al., 1988, Nishimura et al., 2011, Hamilton et al., 2012). IAV then screens the cell surface for a suitable sialylated receptor by using the sialidase activity of NA to eliminate local SAs and release non-productive HA associations (Sakai et al., 2017). It is generally believed that the HA of avian IAV has high specificity for receptors with α-2,3-linked SAs that possess a “linear” appearance (Nobusawa et al., 1991, Rogers and Paulson, 1983). In contrast, HAs from human IAVs prefer an α-2,6 linkage, which causes a more “bent” presentation (Matrosovich et al., 2000, Gambaryan et al., 1997). While these preferences relate to SA linkages in the respective hosts (Debierre-Grockiego et al., 2019), numerous studies have revealed that the HA receptor binding preference with the host SA linkages is not necessary for infection but is more crucial for virus transmission (Tumpey et al., 2007, Herfst et al., 2012, Imai et al., 2012, Linster et al., 2014).

Virus entry through endocytosis occurs via the clathrin-dependent pathway involving dynamin and the adaptor protein Epsin-1 (Roy et al., 2000, Rust et

al., 2004, Chen and Zhuang, 2008). Within the endosome, the acidic pH triggers the M2 ion channel (Lakadamyali et al., 2003, Rust et al., 2004, Pinto and Lamb, 2006) and causes a significant change in HA that reveals the fusion peptide (White et al., 1982, Yoshimura and Ohnishi, 1984, Bullough et al., 1994). When the M2 ion channel is opened, the interior viral particle becomes acidic, freeing the packaged vRNPs from M1 and enabling their transfer to the host cytoplasm following HA-mediated fusion. (Martin and Heleniust, 1991, Bui et al., 1996).

Fusion of the viral and endosomal membranes by HA takes place via several steps (Harrison, 2015, White and Whittaker, 2016) and needs cleavage of HA0 via host cell proteases into two subunits, HA1 and HA2 (Klenk et al., 1975, Lazarowitz and Choppin, 1975, Bottcher-Friebertshauser et al., 2014). The cleavage is required to expose the fusion peptide on the N-terminus of the HA2 after the pH change in the endosome (Bullough et al., 1994). After exposure, the fusion peptide incorporates into the endosomal membrane, whereas the C-terminal transmembrane domain (TMD) attaches HA2 to the viral membrane, forming a pre-hairpin structure. The hairpin bundles later fold into a six-helix bundle allowing the formation of the lipid stalk and the fusion of the two membranes (Matsuoka et al., 2013).

Proteolytic cleavage of HA is essential for influenza virus infection (Klenk et al., 1975) and has been shown to occur on membranes within the Golgi

apparatus (Klenk et al., 1974) or at the cell membrane (Compans, 1973) for HPAI and LPAI, respectively. The polybasic HA cleavage site (pHACS) motif of HPAI is effectively cleaved via ubiquitous proprotein convertases, particularly furin, causing systemic replication in many tissues (Nakamura et al., 2008, Wibawa et al., 2013). Trypsin and trypsin-like proteases cleave the HACS motif of LPAI (Klenk et al., 1975, Bottcher et al., 2006). Thus, LPAI replication is mainly limited to the epithelium of the respiratory and gastrointestinal tract resulting in mild infections of mucous membranes in chickens (Swayne, 2007). Cleavage of LAPIV by trypsin and trypsin-like proteases is facilitated by the presence of Arg or Lys in the HACS motif (Kawaoka and Webster, 1988, Bogs et al., 2010).

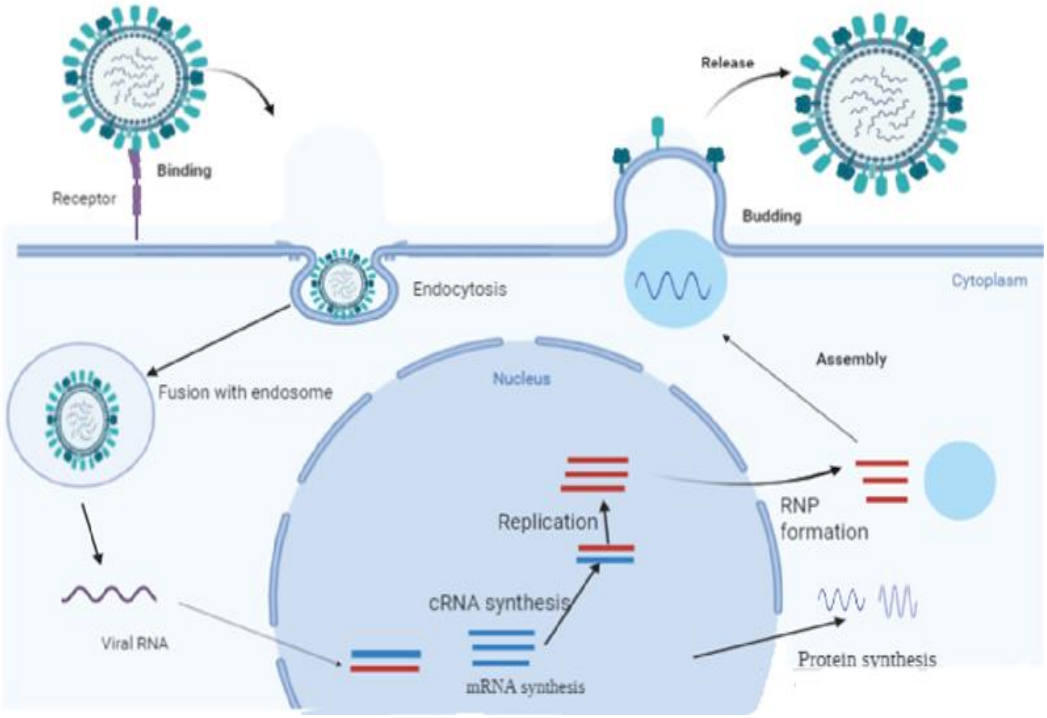


Figure 1.3. The life cycle of the influenza viruses. The figure was adapted from Scolari et al. (2009) and created with BioRender.com.

1.3.3.2. Transcription, replication, and protein synthesis

The transcription of IAV occurs in the nucleus and is facilitated by the viral polymerase. Viral RNA (vRNA) replication begins with the synthesis of a positive-sense copy of the vRNA, known as complementary RNA (cRNA), which is generated to start the replication process (Resa-Infante et al., 2008). This cRNA is transcribed to produce a large quantity of vRNA (Neumann et al., 2004, Palese and Shaw, 2006).

vRNA transcription is triggered by the attachment of PB2 to the 5′-cap structure of host mRNA molecules, in a process known as “cap snatching (Ulmanen et al., 1981, Blaas et al., 1982, Blass et al., 1982). The endonuclease activity of PA (Dias et al., 2009) snatches the cap structure, which acts as a primer for viral mRNA synthesis. Viral mRNA synthesis is conducted by PB1 polymerase activity (Braam et al., 1983).

Two IAV mRNAs (from the segments M and NS) are spliced to generate M1 and M2 or NS1 and NEP proteins. Splicing is conducted by the splicing system of the host but is probably controlled by NS1 (Fortes et al., 1994, Park and Katze, 1995), which attaches to many cellular splicing elements, such as U6 tiny nuclear RNAs (Lu et al., 1994, Wang and Krug, 1998) and UAP56, a splicing element included in the development of the spliceosome (Momose et al., 1996, Momose et al., 2001).

IAV mRNAs are translated by the translation machinery of the host cells, and, not unexpectedly, many cell translation elements, including eIF4A (eukaryotic initiation factor-4A), eIF4E, and eIF4 G, associate with viral mRNAs and polymerase complexes (Burgui et al., 2003, Bier et al., 2011, Yángüez et al., 2011, Yángüez et al., 2012). After infection with IAVs, host cell protein production is reduced, and IAV mRNAs are usually translated (Katze et al., 1984, Katze et al., 1986a, Katze et al., 1986b). In fact, 'cap-snatching' can deplete freshly synthesised nuclear mRNAs from their cap structures, leading to degradation before nuclear export. The association of NS1 with PABII cell (poly(A)-binding protein II) (Burgui et al., 2003, Bier et al., 2011) and cellular CPSF (cleavage and polyadenylation specificity factor) proteins (Chen et al., 1999, Chen and Krug, 2000), and association of the viral polymerase with the C-terminal domain of the main cellular DNA-dependant RNA polymerase II (Pol-II) (Engelhardt et al., 2005, Engelhardt and Fodor, 2006) can lead to prevention of mRNA synthesis in the host (Hutchinson and Fodor, 2012).

Viral polymerase subunit proteins and NP are transported from the cytoplasm to the nucleus via their nuclear localization signals (NLS) (Wang et al., 1997, Bullido et al., 2000, Resa-Infante et al., 2008, Hutchinson et al., 2011) to facilitate the synthesis and transcription of vRNA. Moreover, the M1 (Martin and Helenius, 1991b, Ye et al., 1995), NEP (Greenspan et al., 1985), and NS1 (Greenspan et al., 1988) proteins are transferred into the nucleus

to perform their functions in vRNP nuclear export (M1 and NEP) or the export of cellular and viral mRNAs (NS1) (Schneider and Wolff, 2009).

Nuclear export of freshly synthesised vRNP complexes needs viral NEP (O'Neill et al., 1998, Neumann et al., 2000, Iwatsuki-Horimoto et al., 2004, Cao et al., 2012) and M1 proteins (Martin and Helenius, 1991a, Bui et al., 2000, Chase et al., 2011).

Upon their synthesis by cell translation systems, viral HA and NA proteins penetrate the ER, where they are glycosylated (Klenk et al., 2002, Wagner et al., 2002).

1.3.3.3. Virus packaging, budding, and release

HA and NA are associated with lipid rafts from the plasma membrane (regions abundant in sphingolipids and cholesterol), which are the sites of the budding of IAVs (Kundu et al., 1996, Scheiffele et al., 1997, Lin et al., 1998, Barman and Nayak, 2000, Zhang et al., 2000, Barman et al., 2001, Takeda et al., 2003, Barman et al., 2004, Leser and Lamb, 2005). Segment-specific packaging signals in vRNAs are needed to assemble virions containing eight vRNPs (Fujii et al., 2003, Hutchinson et al., 2010). The M1 protein can play a significant role in the assembly as it associates with lipid membranes (Enami and Enami, 1996, Zhang and Lamb, 1996, Ruigrok et al., 2000); vRNPs (Ye et al., 1999, Baudin et al., 2001, Huang et al., 2001) (Cros and Palese, 2003, Nayak et al., 2004), and NEP (Yasuda et al., 1993, Akarsu et al., 2003). Furthermore, data shows that the M2 cytoplasmic tail

can facilitate vRNP incorporation into the assembling virus particle (McCown and Pekosz, 2005). The enzymatic function of the viral NA protein eliminates SA from host cells and glycoproteins on virions, enabling the release of the virus and avoiding virion aggregation (Figure 1.3) (Palese, 2007, Wright, 2007).

Multiple post-translational alterations have been recognised for IAV proteins, involving HA glycosylation (Webster and Rott, 1987, Palese, 2007) and NA glycosylation (Enami and Enami, 1996). In addition, phosphorylation of M1 (Gregoriades et al., 1990, Whittaker et al., 1995) and NP (Sugiyama et al., 1976, Arrese and Portela, 1996, Bullido et al., 2000) can alter vRNP nuclear import and export (Martin and Helenius, 1991a, Neumann et al., 1997, Bui et al., 2000, Bui et al., 2002). Although the mechanism is not yet fully understood, phosphorylation of NS1 (Hsiang et al., 2012) and PB1-F2 influences virulence (Mitzner et al., 2009).

Glycosylation of HA and NA happens by binding the oligosaccharide molecule to the side-chain amide nitrogen of asparagine (N) in the form of conserved N-linked glycosylation (NLG) (Kornfeld and Kornfeld, 1985). This binding happens with the support of glycosyltransferases and oligo saccharyltransferase in the endoplasmic reticulum (ER) and is accompanied by a complicated trimming phase and remodelling of oligosaccharides via glycosidases and mannosidases during transport through ER and Golgi

apparatus. This phase leads to glycoproteins whose branched NLG structures terminate in mannose (high mannose glycans) (Basak et al., 1981, Ward and Dopheide, 1981). Unlike cellular proteins, complex oligosaccharides on the HA and NA IAVs are not commonly sialylated because NA destroys SA terminals (Basak et al., 1985). NLG is the only form of glycosylation detected for the HA and NA IAV (York et al., 2019).

1.3.4. Host range and cell receptors

There are 18 subtypes of HA and 11 subtypes of NA, which are expressed as H1–H18 and N1–N11 (Webster and Govorkova, 2014). Of those subtypes, the first 16 HA and 9 NA were detected in waterfowl, and the last two were recorded in bats (Tong et al., 2012, Tong et al., 2013). Frequently, influenza strains produce no disease in ducks or other waterfowl. The long-standing balance between AIVs and the duck was interrupted by the appearance of H5N1 viruses (Sturm-Ramirez et al., 2005). HPAI H5N1 was first reported in Egypt in 2005 as an infection in farmed chicken flocks and the virus continues to spread and reach the human population. In 2015, the largest number of human H5N1 cases and deaths attributed to any single country since became endemic in Egypt. Since November 2016, 356 reported human H5N1 infections have occurred in Egypt, of which 119 (33%) have been fatal (WHO, 2017). Moreover, low-pathogenicity avian IAVs, such as H9N2 (Lin et al., 2000) and H7N2 (Belser et al., 2008), produce zoonotic infections (Butt et al., 2005a).

IAV receptors on host cells are glycosylated oligosaccharides terminated by residues of SA attached to glycans via a sialyltransferase-mediated association of SA α 2,3 or 2,6 Gal linkages, expressed in a cell and species-specific manner. AIVs have been realised to attach to SA receptors that are bound to galactose through an SA α 2,3 Gal linkage (SAα2,3-Gal). In contrast, human and classical swine viruses prefer receptors with an SA α 2,6 Gal linkage (Gagneux et al., 2003, Matrosovich et al., 2004). The expression of the proper host cell receptor to which viral HA will attach is a critical determinant of the capability of IAVs to penetrate cells. Consequently, a significant challenge that IAVs need to overcome while passing the species barrier is developing the capability to utilise different host cell receptors (Ito et al., 2000). Chicken cells do, however, contain SA α 2,6 Gal-terminated receptors identified by human viruses. This may explain why certain H9N2 viruses circulate in chickens despite their human-like receptor specificity. Duck influenza virus attaches to gangliosides with short sugar chains, which are plentiful in the intestine of the duck. Human and chicken viruses do not adhere to these gangliosides but show greater attachment than duck viruses to long-chain sugar gangliosides such as those in chicken intestinal tissues (Gambaryan et al., 2003).

Ducks and chickens are the main terrestrial hosts for many IAVs. They are critical natural models for influenza research, with ducks showing tolerance to infection, while chickens, on the other hand, display comparatively high susceptibility. Receptors are essential determinants of viral entry; thus,

variations in receptor distribution between hosts may explain variations in susceptibility to disease, involving contrasting results after infection with several HPAI strains. Among IAV receptors in chickens and ducks, virus attachment studies utilising extracted total gangliosides from intestinal and respiratory epithelial cells plasma membranes have been previously reported (Gambaryan et al., 2003). Based on the attachment of the human influenza virus to the SA α 2,6 Gal receptor specificity, it was observed that chicken epithelial cells express SA α 2,6 Gal receptors. Chicken duodenum and trachea have been studied utilising lectin-binding assays accompanied by immunohistochemistry (Wan and Perez, 2006). There was a notable variation between the primary receptor type in the chicken and duck trachea. In chicken trachea, SAα2,6-Gal was the predominant receptor type, while SA α 2,3 Gal receptors were the most abundant in ducks. This indicates that chickens may be more important as an intermediate host to produce influenza viruses with an elevated capability of attaching to SA α 2,6 Gal receptors and, therefore, a higher possibility for human infection. Chicken intestinal and tracheal epithelial cells have expressed a wider variety of SAα2,3-Gal receptors, both β (1-4) GlcNAc and β (1-3) GalNAc subtypes as compared to ducks, which indicate that they can support infection of a broader range of AIVs (Kuchipudi et al., 2009).

Several proteins (for example, butyrophilin-like (BTNL) and defensins) are present in the duck but not in the chicken genome. Thus, it is proposed that

these proteins have affected the divergence of the four avian genomes and the development of their respective immune systems. β-defensins are triggered via influenza virus infection (Chong et al., 2008), and BTNL is included in T-cell activation and infiltration in mammals (Nguyen et al., 2006, D'Aoust et al., 2010). Some of the BTNL genes may be associated with the host immune response to influenza in ducks (Huang et al., 2013a, Huang et al., 2013b).

1.3.5. Pathogenicity of the avian influenza virus

Defined by the pathogenicity of the disease in chickens, HPAI viruses include H5 and H7 subtypes. Infection with these viruses causes up to 100% mortality within poultry species. Low pathogenicity of avian influenza (LPAI) viruses can result in respiratory signs such as ocular and nasal discharge and inflamed infraorbital sinuses (De Jong et al., 2006, Lee et al., 2007). It is recognised that HPAI viruses develop from low-pathogenicity H5 and H7 viruses and that the critical factor determining virus pathogenicity is the HA proteolytic cleavage, which is needed for the IAVs infectivity in chickens (Rott, 1980, Kawaoka et al., 1984, Horimoto and Kawaoka, 1994, Horimoto et al., 1995b, Banks et al., 2001). The polybasic HA cleavage region is caused by several nucleotide insertions and substitutions to produce basic amino acids codons or via cellular or viral RNAs recombinations (Suarez et al., 2004, Pasick et al., 2005, Maurer-Stroh et al., 2013) and is believed to

be the principal HPAI viruses virulence marker (Horimoto et al., 1994, Gohrbandt et al., 2011).

Most HPAI outbreaks are produced only through H5 and H7 IAV subtypes. However, the H9 virus has gained high pathogenicity in chickens by introducing a pair of dibasic amino acid residues at the HA cleavage region and consequent passages in chickens (Soda et al., 2011). Consecutive adenine residues that are usually detected in the vRNA region encoding amino acids at the cleavage region of LPAIV H5 and H7 viruses detected in waterfowl reservoirs are needed for nucleotide insertions and mutations into the vRNA region (Nao et al., 2017).

1.3.6. Genetic variation

Genetic variations take place regularly because of the structure of the segmented vRNA and the reduced proofreading of the RNA-dependent RNA polymerase, which produces replication errors throughout the virus life cycle (Zambon, 1999, Zambon, 2001). Therefore IAVs can experience recurrent genetic modifications that may affect the antigenicity of the virus (Shors, 2016). The modification in HA and NA protein structure may enable the virus to avoid neutralising antibodies, the critical method of protective immunity against IAV infection. Such modifications may cause the formation of a virus

strain distinctive from formerly circulating viruses (Zambon, 1999, Smith, 2001).

1.3.6.1. Antigenic shift

Antigenic shift is defined as a sudden change in IAV surface proteins (principally HA) due to the reassortment of the viral genomes of two different IAV strains. This may introduce a new virus into the human population with little or no immunity. Therefore, everyone is susceptible to infection, and widespread epidemics or pandemics may result. The cause of these new strains is mainly AIVs that are found naturally in migratory wild birds (the natural reservoir) (Khan, 2009, Parija, 2014). The antigenic shift may occur via genetic reassortment, direct transmission, adaptation, or by re-introducing old (previously circulating) virus subtypes. Genetic reassortment may happen between two different strains of IAVs in a single host cell, wherever the RNA segments from the two strains are mixed through new virus reassembly. Therefore, a third viral strain will appear that includes the merged genes of both strains (Parija, 2014). Pigs have α 2, 3, and α 2,6 linked SA receptors in their respiratory tract so that, in theory, they can simultaneously act as hosts for both AIV and mammalian virus strains, causing mixed gene strains to emerge; they are often described as “mixing vessels.” 'Asian flu' in 1957/1958, 'Hong Kong flu' in 1968/1969, and pandemic swine H1N1 in 2009/2010 are believed to have been produced by genetic reassortment between avian and mammalian viruses.

Direct transmission and adaptation: the direct transmission of IAVs, mainly with an HPAIV strain, may occur from birds to humans. The virus may then adapt further in humans. This adaptation has happened via modifying the receptor binding region of HA from SA α 2,3 Gal linked receptors in avian cells into SA α 2,6 Gal linked receptors in human cells by mutation (Sleman, 2017). Consequently, a new human strain might be generated and be able to spread within the population, leading to a pandemic. Re-emergence of old strains: once the immunity of the population has faded to a specific strain, the virus can re-emerge, leading to a pandemic outbreak within that population. For instance, the "Russian flu" in 1977 was a re-emergence of an H1N1 virus subtype that had previously circulated in 1950 (Garten et al., 2009, Parija, 2014).

The main site of influenza virus replication in the human host is the upper respiratory tract which has plenty of SA of the α 2,6 isomer, while the avian digestive system is the leading replication site in the avian host, which contains the α 2,3 isomer (Shinya et al., 2006, Pillai and Lee, 2010). Mutation in the receptor-binding of the HA can allow for efficient SA α 2,6 Gal rather than SA α 2,3 Gal binding (Matrosovich et al., 2004, Pappas et al., 2010). This has not been observed for the H5N1 subtype but has happened for some H9 mutant strains (Wan et al., 2008, Song et al., 2009) (Shelton et al., 2011).

1.3.6.2. Antigenic drift

Antigenic drift is the antigenic change of influenza viruses owing to repeated spontaneous point mutations (Figure 1.4) (Davidson et al., 1988).

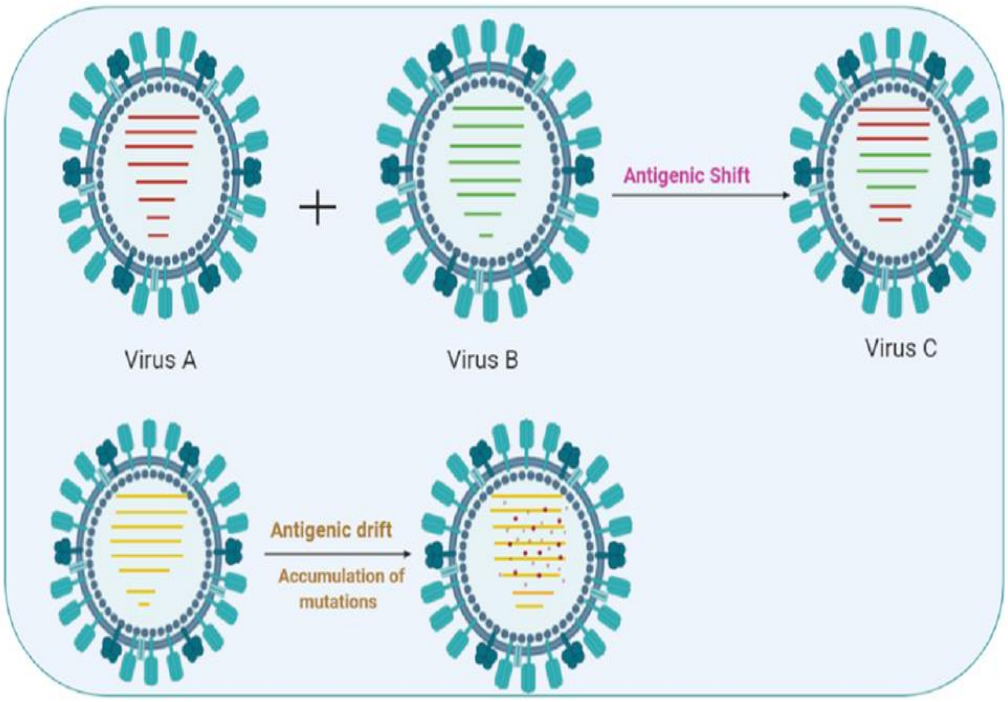


Figure 1.4. Antigenic shift and antigenic drift in the influenza virus. Figure adapted from Rajaram et al. (2020) and created with BioRender.com.

The procedure of antigenic drift is gradual, including point mutations within antibody-binding regions in the HA, the NA, or both, which may happen every time the virus replicates (Fitch et al., 1997, Suarez et al., 1999, Suarez and Senne, 2000, Stallknecht, 2003). Most of the mutations are insignificant because they do not affect protein conformation; however, some mutations lead to viral protein modifications, which reduce the attachment of host antibodies. Multiple mutations in H5N1 viruses in Egypt have led to antigenic

drift, affecting major antigenic sites in the receptor binding domain (RBD) (Cattoli et al., 2011). Genetic reassortment has been detected in Egyptian H9N2 viruses. Antigenic analysis showed that antigenic drift found mainly among H5N1 and H9N2 viruses are homogeneous in Egypt (Kandeil et al., 2019).

The HA of HPAIV H5 contains four amino acid modifications (S123P-T156A-D183N-S223 R) in the head of the HA that dramatically affect the NA preference of the H5 gene of clade 2.3.4 viruses. The T156A substitution, which led to the NA shift among the documented amino acid substitutions, also significantly altered the H5N1 virus antigenicity, indicating an occurrence of antigenic drift (Antigua et al., 2022).

1.3.7. Mode of transmission

Three IAV transmission modes have been recognised: droplet, airborne, and contact transmission (Garner, 1996, Nicas et al., 2005, Tellier, 2006, Brankston et al., 2007). Droplet transmission requires the infectious case to directly spray droplets onto the mucous membranes of a vulnerable host by coughing or sneezing. Droplet nuclei allow for airborne transmission that does not require direct interaction with the infectious case. The virus can be directly transmitted into the alveolar region by droplet nuclei, which descend slowly from the air and are respirable. The influenza virus can stay alive for several hours (Weber and Stilianakis, 2008).

Contact transmission happens directly via physical contact between a susceptible host and an infected individual or indirectly via contact with secretions on fomites (Garner, 1996, Boone and Gerba, 2007).

Some viruses may create species-specific permanent lineages of IAV and cause epidemics in the new host during adaptation via mutation or genetic reassortment. In the 20th century, developing these lineages in the human population resulted in influenza pandemics. Additionally, the transmission of viruses and transient infections may take place between the new hosts, such as pigs or chickens and humans (Rogers and Paulson, 1983, Rogers and D'Souza, 1989, Gambaryan et al., 1997, Matrosovich et al., 1997, Matrosovich et al., 2004).

Reservoir hosts, such as waterfowl, infected with AIVs discharge vast quantities of the virus in faeces and other secretions, polluting the natural environment, such as dust, cages, and soil. AIVs may remain infectious in soil or contaminated equipment for a long time, depending on the humidity and temperature (extended in cold climates). Disease in birds produced by highly pathogenic AIVs leads to systemic replication and the existence of the infectious virus in their eggs and many tissues. Most human infections with AIVs included contact with affected poultry or live poultry the week before the beginning of the disease (Mounts et al., 1999, Koopmans et al., 2004, Tran, 2004). Exposure to contaminated water is considered the most vital

transmission route among aquatic birds (Katz et al., 1999, Koopmans et al., 2004, Ungchusak et al., 2005).

1.4. Clinical signs of avian influenza virus

Several countries in the Middle East, Africa, Asia, and Europe experienced a widespread spread of the poultry-adapted H9N2 virus in the late 1990s. H9N2 LPAI infections in chickens can result in mild to severe respiratory disease symptoms, substantial economic loss from decreased egg production, increased morbidity rates, and up to 20% mortality (Pusch and Suarez, 2018). H9N2 LPAI infections can increase the severity of clinical signs and mortality in infected birds (Samy and Naguib, 2018, Erfan et al., 2019). LPAI usually results in respiratory signs, e.g. coughing, sneezing, and nasal discharge. Sinusitis is usual in domestic ducks, quail, and turkeys. In breeders and layers, there may be infertility or reduced egg production. The mortality rate is usually low unless accompanied by secondary viral or bacterial infection. LPAI H9N2 is the most frequently isolated subtype in bird markets in Asia, North Africa, and the Middle East (Swayne, 2019).

AIV strains that rapidly kill birds may have less obvious clinical signs (Alexander et al., 1978, Bean et al., 1985, Perkins, 2001). Chickens infected with HPAI viruses are occasionally noticed dead without previous signs (Alexander et al., 1978, Bean et al., 1985, Forman et al., 1986, Elbers et al., 2004, Nakatani et al., 2005, Swayne, 2007, Tsukamoto et al., 2007).

HPAI viruses can produce severe disease with mortality of up to 100% in a short period in poultry. Clinical signs might not appear in peracute cases before death. However, in severe cases, lesions may include oedema, cyanosis of the comb, head; bloody nasal discharges; and subcutaneous haemorrhages (Swayne, 2019).

1.5. Laboratory diagnosis

Laboratory diagnosis is vital to identify IAV infection in seasonal outbreaks or pandemics (Dwyer et al., 2006). Laboratory examinations should distinguish them from flu-like diseases produced by other respiratory viruses and bacterial organisms (Allwinn et al., 2002, Patrick and Richard, 2003). Several diagnostic methods for AIV infection have been applied and are categorised into direct and indirect methods. The direct techniques involve direct detection of viruses and quick antigen detection such as ELISA. Additional studies on the influenza virus have been achieved by quantitative reverse transcriptase-polymerase chain reaction (RT– qPCR) and serological tests (neutralization tests and haemagglutination inhibition (HI). These tests have specific advantages and drawbacks with varying sensitivity rates (Dwyer et al., 2006). The indirect methods include conventional cell culture, egg culture, or laboratory animal inoculation for propagation as well as typing of the viruses. Embryonated hens' eggs are frequently used for growing virus isolates, thereby increasing virus titre (Dwyer et al., 2006, Khanna et al., 2008). RT–PCR is commonly more sensitive and is time-

saving. It gives accurate results and eases the typing and subtyping of AIVs (Ellis et al., 1997).

Moreover, multiplex PCR can be utilised to confirm the infection by involving a universal primer set to detect the existence of multiple genome segments in the same reaction (Hoffmann et al., 2001, Ellis and Zambon, 2002). This method is frequently used to identify the viral M gene, which is the most conserved gene among all IAV subtypes (Spackman and Suarez, 2008). AIVs can be isolated via cell culture methods where a specimen is injected into a live culture system, and the virus is next detected after a specified incubation period. Embryonated hens' eggs, 10 to 12 days old, are often used to isolate and grow the influenza virus via injecting the allantoic cavity with the specimen. It commonly requires 3 days or more to amplify the viruses in the eggs before harvesting (Wang and Taubenberger, 2010).

1.6. Immunity to influenza A virus

1.6.1 Innate immunity

The first phase of host defence against IAV infection is the innate immune reaction, triggered shortly after infection. There are three main components utilised by the host: antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages and monocytes, pattern recognition receptors (PRRs), and cytokines (Kreijtz et al., 2011).

Macrophages are among the primary immune cells in the innate immune system that recognise and remove numerous pathogens. Microbial

recognition by macrophages is mediated by macrophage-expressed receptors known as PRRs, including several toll-like receptors (TLR) (Medzhitov and Janeway, 1997, Akira, 2003, Meylan et al., 2006). After virus infection, the TLR triggers downstream adaptor molecules stimulating intracellular signalling cascades (Sasai and Yamamoto, 2013) with a significant upregulation of the gene transcription of the pro-inflammatory molecules. The stimulated pro-inflammatory mediators include cytokines (e.g., interleukin (IL)-1β) and inducible nitric oxide synthase (iNOS) (Uematsu and Akira, 2007, Haddadi et al., 2015, Abdul-Cader et al., 2016). iNOS will enhance the formation of a highly reactive antiviral free radical molecule, nitric oxide (NO), as a part of the innate host defence (MacMicking et al., 1997, Abdul-Cader et al., 2017). Interferons generated from these cells can stimulate DCs resulting in the activation of antigen presentation to $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells, which further promotes the adaptive immune response (Kreijtz et al., 2011).

IAV infection can be identified by certain PRRs, including some TLRs, melanoma differentiation-associated gene 5 (MDA5), and NOD-like receptors (NLR). These receptors identify pathogen-associated molecular patterns (PAMPs), and NLR may also affect virus replication (Pang and Iwasaki, 2011). Of the 11 identified TLRs, TLR–3 and TLR–7 have been revealed to play a significant role in detecting double-stranded and single-stranded viral RNAs, respectively. MDA5 has been demonstrated to bind

double-stranded RNAs. Signalling by such receptors results in the generation of inflammatory cytokines from affected host cells, including IFN, TNF, and macrophage inflammatory protein (MIP) produced by natural killer (NK) cells (Alexopoulou et al., 2001, Lund et al., 2004, McGill et al., 2009). Such cytokines have an antiviral effect where they can destroy virus-infected cells and prevent viral replication. This immunity is the starting point for cellular immunity to reduce virus replication (Achdout et al., 2003).

While the host organism produces antiviral responses to kill new viruses after infection, IAV may escape these reactions by developing various immune evasion approaches to propagate effectively. This includes binding IAV proteins to different components of the innate immune response. For instance, the NS1 protein plays a significant role in preventing the release of IFN. PB2 and PB1-F2 inhibit the generation of IFN-β by interaction with mitochondrial antiviral signalling protein (MAVS) (Van de Sandt et al., 2012). The study revealed that autophagy, an effective defence against IAV infection, is mediated via the virus through the association of M2 with the vital autophagy protein, microtubule-associated protein 1A/1B – light chain (LC3). Such association subverts autophagy and facilitates the relocation of LC3 to the plasma membrane of virus-infected cells. This may generate sufficient support for viral budding and improve the viability of filamentous viruses (Beale et al., 2014).

1.6.2 Adaptive immunity

Protective immunity can be achieved by generating humoral immunity, cellular immunity, or both.

1.6.2.1 Humoral immunity

Humoral immunity is a particular form of immune response that tends to block entry of the pathogen through the production of antibodies by B cells and often inhibits reinfection of hosts previously infected with the same strain or similar virus strain (Hampson and Mackenzie, 2006). HA antibodies are well known to mediate protection against IAV. Antibodies attach to the HA and prevent virus attachment to the host cell (Kreijtz et al., 2011). Antibodies to NA can reduce the infection spread by preventing the enzymatic activity of NA (Hampson and Mackenzie, 2006). Both proteins have high sequence variability resulting in antigenic differences between subtypes. Therefore, protection provided by humoral immunity is subtype-dependent and strain-dependent and is inefficient against different subtypes of HA and NA or rapidly evolving viruses. This explains why influenza vaccines must be modified regularly to produce humoral protection against HA molecules of newly evolving viruses. Antibodies to M2 can influence virus neutralisation, although to a minimal extent relative to antibodies for HA and NA (Suarez and Schultz-Cherry, 2000a, Chen and Deng, 2009).The main groups of immunoglobulins (Ig) produced after IAV infection are mucosal antibodies (IgA) and circulatory antibodies (IgM and IgG). They also have a main

function in virus neutralisation and removal (Mazanec et al., 1995). Humoral immunity in poultry following typical infection involves the production of systemic and mucosal antibodies. The circulatory antibody reaction is assessed by IgM development after five days of infection. IgY, like mammalian IgG in function but somewhat variable in composition, is found shortly after that. Antibodies that attach to surface viral proteins (HA, NA, and M2) are necessary for disease prevention (Suarez and Schultz-Cherry, 2000a).

1.6.2.2 Cellular immunity

$CD4^+$ and $CD8^+$ T lymphocytes can be stimulated through infection. APCs display MHCII on their surfaces. Once the virus is engulfed, the lysosome carries the digestive enzymes associated with the phagosome to process the antigens. The processed antigens are combined with the MHC II proteins forming the MHCII antigen peptide complex and are presented on the surface of the APCs. Helper T cells ($CD4^+$) identify the antigen and bind to the complex. T helper cells (Th) are classified into subsets, including Th1 and Th2. Th2 cells secrete cytokines that help enhance humoral immunity (Soghoian and Streeck, 2010). Th1 cells, on the other hand, facilitate the production of cellular immunity by activation of $CD8^+$ lymphocytes. Peptides are transferred to the ER, where they are combined with MHC I, and then this complex (peptide / MHCI) is transferred to the plasma membrane through the Golgi apparatus. $CD8^+$ cells (cytotoxic T cells) identify this

complex as abnormal, triggering these cells that finally lyse virus-infected cells (Yewdell et al., 1985).

Cellular immunity gives only limited protection against HPAI disease in poultry. HPAI-induced mortality is rapid within one week, insufficient to trigger cytotoxic T-lymphocyte (CTL)-specific immune response to fight the disease; inactivated AIV vaccines promote memory T lymphocytes and humoral immunity. However, a few studies have revealed that cellular immunity can decrease the severity of HPAI disease in chickens and turkeys. $CD8^+$ CTLs lymphocytes have been shown to play a critical role in regulating lung respiratory viruses (Allan et al., 1990). $CD8^+$ CTLs targeting viral epitopes conserved in IAVs, such as those between HA and NP, have been correlated with protection against IAVs (Anderson et al., 1992, Altstein et al., 2006). In other studies, NP-specific CTLs generated by live virus vaccines led to protection from the lethal challenge and supported faster virus elimination in mice (Ada and Jones, 1986, Andrew et al., 1986). Only a few studies have evaluated the CTL reactions in poultry to AIVs. Seo and Webster (2001) reported that spleen-derived T lymphocytes or $CD8^+$ cells, produced after infection with H9N2 AIVs, may provide partial protection against subsequent lethal H5N1 HPAI virus challenge following their transfer to naive MHC-matched birds (Swayne and Kapczynski, 2008).

1.7. Vaccination against influenza A virus

Although antiviral drugs are a vital part of public health measures for the treatment of influenza, vaccination is the most effective way to protect public health in a pandemic. The accessibility of vaccines through a pandemic could end zoonotic transmission and inhibit the advance of new cases. The presence of safe and effective, widely acting, or universal vaccines would be highly valuable to reduce transmission and, consequently, disease and mortality due to a pandemic with a novel IAV (de Vries et al., 2018).

Vaccination against AIV is important for controlling the disease in poultry and stopping the virus from spreading to humans. AIV vaccines are commonly used in countries where AIV is endemic, such as Egypt, China, Vietnam, and Indonesia (Swayne et al., 2011, Swayne and Spackman, 2013, Kim and Samal, 2019).

IAV vaccines may be classified into six groups: (1) inactivated influenza vaccine, (2) live attenuated vaccine, (3) subunit vaccine, (4) vector-based vaccine, (5) nucleic acid vaccine, (6) virus-like particle (VLP) (Table 1.3).

First and second vaccines, the LPAI virus isolated through poultry epidemics or from wild or domesticated bird screening, has become the principal virus strain used in inactivated AIV vaccines. Generally, these viruses have been cultivated in 9–11-day-old embryonated hens' eggs, the allantoic fluid collected, and the virus inactivated utilizing chemicals (formalin, propiolactone). With the development of reverse genetics (Fodor et al.,

1999, Neumann et al., 2005), using such clone systems is possible, generating inactivated vaccines (Liu et al., 2003, Lee et al., 2004). The field strains can be modified by reverse genetic techniques to be safe by altering the HA cleavage site from HPAI to LPAI. AIV vaccines utilising the oil adjuvants method benefit from generating high antibody titres in poultry, and immunity against homologous HA field strains is excellent over long periods (Swayne et al., 1999). However, the utilization of inactivated AIV vaccines is confined due to high labour costs for parenteral administration and the difficulties in detecting infected birds with traditional serological testing in the vaccinated community, e.g., differentiating infected from vaccinated animals (DIVA). Specific serological tests for the DIVA strategy need to be established. The immune reaction depends on the quantity of antigen in each dosage and adjuvant used (Swayne, 2009).

Live attenuated influenza vaccines provide significant benefits compared to inactivated ones. Oral administration of live attenuated vaccines leads to the activation of the virus in epithelial cells and induces a robust immune response that provides immunity against distant clade viruses (Kreijtz et al., 2007). Live attenuated influenza vaccine has many benefits over inactivated influenza vaccine. These vaccines can be generated safely and cheaply (Spackman and Swayne, 2013, Jiang et al., 2014, Li et al., 2014). The immunity elicited against live vaccines includes both humoral and cellular immunity against viruses propagating in relevant tissues (Spackman and Swayne, 2013). Currently, many established methods are being used to

produce live attenuated influenza vaccines, but the main disadvantage is that the live attenuated vaccine can revert to virulence (Elizaveta and Alexandra, 2018).

Third, significant amounts of viral proteins, e.g. HA or NA, can be produced in an *in vivo* system, and this crude or purified HA can be integrated into the vaccine. This can be achieved by introducing the HA gene into eukaryotic cell cultures, yeat, and plants (Saelens et al., 1999), bacterial (e.g., Escherichia coli) (Davis et al., 1983), or viral vectors (e.g., baculovirus) (Crawford et al., 1999), and the use of industrial methods to generate avian influenza viral HA, accompanied by oil emulsification (Swayne, 2009). Subunit vaccines are easy to produce and safer than live vaccines. However, subunit vaccines have poor immunogenicity and need adjuvants to boost the immune response (Tregoning et al., 2018, Khalaj-Hedayati et al., 2020).

Fourth, protecting antigens may be expressed in *vivo*, e.g. by utilising live administered viral or bacterial vectors of nucleic acid inserts of the HA gene as either cDNA or RNA. Such vectored vaccinations use a fowl poxvirus (Taylor et al., 1988), vaccinia virus (Chambers et al., 1988), Newcastle disease virus (Swayne et al., 2003, Park et al., 2006, Veits et al., 2006), herpesvirus or infectious laryngotracheitis virus (ILTV) backbone (Veits et al., 2003).

The recombinant fowlpox-AI-H5 (rFP-AIV-H5) is the leading vector technology used in approved AIV vaccines. RFP-AIV-H5 has some benefits over inactivated AI vaccines, including DIVA approaches, which are easier to establish as they use standard serological tests to identify HA (HI) antibodies and the absence of NP antibodies (AGID or ELISA) present in AIVs. However, there are two disadvantages to rFP-AIV-H5: first, rFP can be used only in chickens, and second, chickens have to be naive to the fowlpox vector for immunisation to be efficient. The recombinant Newcastle Disease Virus-AI with H5 or H7 avian influenza gene inserts has advantages over both disadvantages above through facilitating mass application (aerosol spray or eye drop) to minimise administration costs, protecting against AIV-specific subtypes and all NDV, and using standard serological testing as a DIVA serological technique. Pre-existing maternal antibodies against the Newcastle disease virus vector or AIVs can inhibit the replication of the vaccine and thereby minimise or eliminate a protective immune response. Eventually, all vaccines must be tested for safety (Swayne, 2009).

Nucleic acid vaccines, mainly as DNA plasmids with cDNA inserts that code for HA, generate protection by a presentation on antigen-presenting cells (APCs) or myocytes and associated immune reactions (Suarez and Schultz-Cherry, 2000b). IAVs HA-based DNA vaccines are effective, although, with existing technology, they are expensive (Suarez and Schultz-Cherry, 2000a, Suarez and Schultz-Cherry, 2000b). $H5_{HA}$ DNA vaccine from

A/Gyrfalcon/Washington/40188-6/2014 (H5N8) is approved for use in the USA (WOAH, 2021).

mRNA vaccine is promising for improved strain match and immune response (Pecetta and Rappuoli, 2022). Recently, quadrivalent modified mRNA encapsulated by lipid nanoparticles with modified HA, NA, M2, and NP from group 2 IAV showed wide spectrum protection against group 2 influenza viruses (McMahon et al., 2022). However, the mRNA vaccines must be stored at -80°C, so transporting this vaccine will be expensive.

Table 1.3. Demonstration of the common vaccination strategies and their respective advantages and disadvantages. The table is adapted from de Vries et al. (2018).

Vaccine	Advantage	Disadvantage	References
Live attenuated vaccine	Contain essential epitopes. Single-dose is enough. Robust cellular immunity.	Safety hazard. Return to wild-type potential. Intervention via maternal antibodies.	(Suguitan Jr et al., 2006, Karron et al., 2009, Talaat et al., 2009, Wang et al., 2010, Pitisuttithum et al., 2017).
Inactivated vaccine	Safe.	Transient immunity.	(WHO, 2022, Cox et al., 2009, Couch et al.,

	Good humoral immune responses.	Frequent many doses are required. Poor stimulation of cellular immunity.	2012, Krammer and Palese, 2015)
Subunit vaccine	Safe. Choice of antigen potential.	Poor immunogenicity. Poor stimulation of cellular immunity.	(Stephenson et al., 2003, Levine et al., 2017, Sun et al., 2017).
DNA vaccine	Contain essential epitopes. Choice of antigen potential. Stimulation of cellular immunity.	Poor protective immunity when used alone.	(Laddy et al., 2007, Laddy et al., 2008, Rao et al., 2008, Laddy et al., 2009, Smith et al., 2010).
Virus-like particle	Safe. Contain essential epitopes.	Complex production procedure.	(Giles and Ross, 2011, Crevar et al., 2015, Ren et al., 2015).
Vector-based vaccine	Safe. Contain essential epitopes. Choice of antigen potential.	Multiple doses are needed. Interference by vector-specific immunity.	(Kreijtz, 2009a, Kreijtz, 2009b, Altenburg, 2014, Kreijtz, 2014, Webby, 2015).

	Stimulation of cellular and humoral immunity. Efficient in heterologous prime-boost.

The following vaccine types are commercially available in chickens; monovalent inactivated vaccines involving either H7 or H5 strains, bivalent involving H7 and H5 strains, and monovalent and bivalent vaccines with homologous or heterologous NA subtypes. Recombinant viral vectored vaccines (fowlpox H5) are effective only in chickens and are only used in day-old chicks before any contact with wild-type fowlpox virus, which would stop the effectiveness of the vectored vaccine (OIE/FAO/IZSVe, 2007).

1.7.1. Plant-based vaccines

Despite the existence of commercial vaccines for significant viral diseases in poultry, such diseases still produce substantial losses. There are several reasons for this: the vaccines may be expensive, storage of attenuated virus vaccines in the cold chain might not be achievable, and commercial vaccines may inadequately protect against local circulating strains (Kandeil et al., 2019).

There is an immediate need for vaccines that can be developed at reasonably low expense, are stable, and can be adapted to local strains of the virus. Plant-based vaccines could provide these benefits and are appropriate for use in developed countries.

VLPs resemble the structure of the virus but are devoid of the inner nucleic acid, so they are safe. VLPs are highly immunogenic because they have a repetitive surface epitope that highly triggers the immune response (Noad and Roy, 2003, Tariq et al., 2021). VLPs are classified as enveloped or non-enveloped VLPs depending on the presence of the lipid envelope (Jeong and Seong, 2017, Tariq et al., 2021). Non-enveloped VLPs are composed of the important nucleocapsid proteins of the virus and can be produced by one or multiple capsid proteins (Naskalska and Pyrć, 2015, Tariq et al., 2021). VLPs composed of multiple capsid proteins are complex and contain many layers. These VLPs d not contain any host proteins and are easy to produce and purify (Lua et al., 2014, Tariq et al., 2021). Enveloped VLPs are composed of the main lipid glycoproteins of the virus, which is the target for the neutralising antibodies. These VLPs contain the host lipid membrane, making them difficult to purify (Kushnir et al., 2012, Tariq et al., 2021).

Furthermore, VLPs may not require adjuvants to provide effective immune activation. The self-adjuvant effects of these VLPs enable their uptake by DCs for the processing and presentation of major histocompatibility complex (MHC) class II and the stimulation of DC maturation and migration, which is

essential for innate immune response stimulation (Fifis et al., 2004, Gamvrellis et al., 2004). VLPs are also processed via the MHC class I pathway, activating CD8+ T-cells, which are crucial for destroying intracellular viruses. Receptor-mediated penetration has been shown to occur through a SA interaction with the HA/NA of influenza viruses (Zeng et al., 2002). Stimulation of DCs liberates cytokines, such as IFN- α/β, which does not require replication (Frank et al., 2002). VLPs have advantages over live attenuated viruses for immune activation because many viruses that multiply in DCs are reported to inhibit cell stimulation and maturation via the expression of specific viral proteins (Teleshova et al., 2003, Grgacic and Anderson, 2006).

1.7.2. Plant-based expression pipelines

One well-developed method of plant expression uses the bacterium *Agrobacterium tumefaciens* to incorporate plasmid encoding of the specified antigen into appropriate plants (e.g., *Nicotiana benthamiana*). Proteins are transiently expressed at elevated rates, adequately glycosylated and folded, and can be easily purified (e.g. via the inclusion of a His-tag and Ni-NTA chromatography) (Takeyama et al., 2015).

New technologies for inserting recombinant genes into plants have also been established. Protocols known as 'agroinfiltration' using pressure (using a syringe or vacuum) to infiltrate the leaves of 6-week-old plants such as *N. benthamiana* with *Agrobacterium,* including binary vectors, have been

developed (Leuzinger et al., 2013). The usage of agroinfiltration in vaccine research was developed by the Canadian biotechnology company Medicago, which produced VLP vaccines for influenza HA antigens (Landry et al., 2010). Unfortunately, Medicago was closed by February 2023. The antigenicity of human and animal viruses is assessed mainly by identifying their surface antigens to obtain protective immunity and antigens are well-presented on the VLPs (Kushnir et al., 2012, Vacher et al., 2013). Agroinfiltration using suspensions of *A. tumefaciens* allows significant quantities of vaccine proteins to be generated within a few days to a few weeks, much quicker than for stable expression systems (Leuzinger et al., 2013).

1.7.3. Plant-based avian influenza virus vaccine trials

The quadrivalent virus-like particle (QVLP) vaccine of human IAV was produced in *Nicotiana benthamiana* via transient expression using an *Agrobacterium* infiltration platform. HA proteins in the VLP were focused on influenza strains recommended by the World Health Organization. The final doses were determined based on the content of HA. At higher doses, the QVLP vaccine induced both homologous and heterologous antibody responses, with the addition of alum having little to no effect. Additionally, the candidate vaccine consistently induced homologous and heterologous $CD4^+$ T cells identified by their generation of interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumour necrosis factor-alpha (TNF-α) (Pillet et al.,

2016). Medicago reported that Health Canada had approved the new drug submission (NDS) for a plant-based QVLP influenza recombinant vaccine for scientific review (Medicago, 2019). The QVLP vaccine reached the phase II safety clinical trial, which showed that the vaccine could protect adults against influenza infection with no significant adverse effects (Ward et al., 2020).

VLPs exhibiting H5N1 (H5) protein on their surface (H5 VLP) caused an extended (up to 6 months) cross-reactive HA-specific $CD4^{+}$T cell immune response in healthy adults. The potential of low-dose adjuvanted H5 VLP to trigger both humoral immunity and persistent cell-mediated immunity in healthy adults is very appealing and may result in substantial dose savings during a pandemic (Pillet et al., 2018).

H5N1 HA1 was transiently expressed in *Nicotiana benthamiana,* tested using western blot and ELISA*,* and found to be expressed in high quantities (Farsad et al., 2017).

Trimerisation and oligomerisation effects on protein immunogenicity were studied. Disulphide bonds were added to plant transiently expressed H5 trimers to produce H5 oligomers, and then the mice were vaccinated with H5 trimers and oligomers. The mice had stronger immunity against H5 oligomers than trimers and were protected from the lethal HPAIV H5N1 challenge (Phan et al., 2020) (Table 1.4).

H6 VLP was transiently expressed in *Nicotiana benthamiana* and used in chicken vaccination. H6 VLP induced a robust immune response, and

chickens were protected against heterologous H6N2 challenge and showed decreased virus shedding (Smith et al., 2020).

The C-terminal isoleucine zipper chicken immunoglobulin (GCN4pII-IgMFc) motif was added to the HA cassette to stimulate the trimerisation of the transiently expressed protein. This motif increased the haemagglutination titre of the recombinant H5 proteins by forming oligomers that enhance immunogenicity (Van et al., 2020).

Trimerisation of proteins was tested, and trimeric HA was synthesised, representing the circulating H5N1 strain in poultry in Vietnam from 2005 to 2015 and transiently expressed in *Nicotiana benthamiana.* The H5 HA proteins induced high neutralising antibodies in mice (Thi Pham et al., 2019). Strep-Tactin® XT and Strep-Tag have been transiently expressed in *Nicotiana benthamiana* and used to generate HA oligomers tested in mice, generating an enhanced neutralising antibody response (Phan et al., 2018). HPAI H5 was transiently expressed in *Nicotiana benthamiana*, and the HA1 was expressed as a soluble protein. Mice were vaccinated with purified HA1 and elicited neutralising antibodies (Pua et al., 2017).

An alternative approach has used seeds to generate recombinant proteins. Two Phaseolus vulgaris seed storage protein gene sequences were added to H5N1 HA and stably expressed in *Nicotiana benthamiana*. The protein content in the seed was high, and proteins remained stable at room

temperature for years. The HA proteins were injected in mice, generating high titres of HA-specific antibodies (Ceballo et al., 2017).

H5N1 HA was transiently expressed in *Nicotiana benthamiana* and added to cyclic dimeric guanylic acid (c-di-GMP) mucosal adjuvant to immunise mice via the intranasal route. The mice produced neutralising antibodies and survived a lethal virus challenge (Major et al., 2015).

The amount of protein expression was recorded using a GUS reporter. The expression of the M2 epitope peptide was tested, and M2e of H5N1 fused to GUS and infiltrated in tobacco. The M2e protein was highly expressed (Tarasenko et al., 2013).

Comparisons between full-length and truncated gene expression were studied. Two variants from H5N1 HA are synthesized: one is the full-length HA, and the other is truncated HA without a transmembrane domain. The HA site accumulation in plants was studied. Two variants of H5 HA full-length and truncated genes) were transiently expressed in *Nicotiana benthamiana* and found that the full-length HA accumulated in the apoplastic space while the truncated one accumulated in the ER. Chicken and mice were immunised with the two variants, eliciting poor neutralising antibodies (Mortimer et al., 2012).

The effect of endoplasmic reticulum retention signal SEKDEL on protein expression levels was studied. H7N7 HA was fused to SEKDEL signal peptide and transiently expressed in *Nicotiana benthamiana* and found that the HA was expressed in high quantities (Kanagarajan et al., 2012).

The effect of human or murine Fc fragment fusion heavy chain was tested. H5N1 HA, either full length or HA1, was fused to His-tag, Myc-tag, or human or animal Fc antibody fragments transiently expressed in *Nicotiana benthamiana.* HA was expressed in high quantities and found to elicit an antibody response after intramuscular immunisation in mice, but none of the HA1 preparations elicited an antibody response in mice. These tags increased protein expression levels, but without affecting the immune response, Fc fragment fusion improved the protein expression levels and facilitated purification (Spitsin et al., 2009).

The effect of the LicKM carrier was studied. LicKM carrier is a modified bacterial carrier from *Clostridium thermocellum.* LicKM carrier has been shown to work as an adjuvant and carrier for the important epitopes to be presented for the immune system. H5N1 HA was fused to the LicKM carrier and transformed into a pBID4 vector, which helps produce highly stable proteins. HA was expressed in high quantities (Musiychuk et al., 2007).

Table 1.4. Trials of avian influenza plant-based vaccine.

Avian Influenza strain	**Target antigen**	**Immunity**	**Notes**	**References**
-A/duck/Vietnam/TG24-01/2005 (H5N1)	HA	Neutralising antibodies in mice and chicken	Expression of oligomeric and trimeric proteins.	Phan et al. (2020)

-A/duck/Vietnam/HT2/2014 (H5N1)				
A/chicken/South Africa/N2826/2016 (H6N2)	HA VLP	Neutralising antibodies in chicken	Challenge experiment in immunised chicken using heterologous virus strain.	Smith et al. (2020)
A/duck/Vietnam/TG24-01/ (2005)	HA	Neutralising antibodies in chicken	Effect of GCN4pII-IgMFc motif on protein expression.	Van et al. (2020)
A/H5N1 avian strains detected from 2005 to 2015 in Vietnam	HA	HA-specific Immunoglo-bulin G (IgG) in mice	Effect of trimerisation on protein immunogeni-city.	Thi Pham et al. (2019)
A/duck/Viet-nam/TG24-01/2005 (H5N1)	HA	Neutralizing antibodies in mice	The use of Strep-Tactin® XT and Strep-Tag for the production of protein	Phan et al. (2018)

			oligomers was studied.	
A/chicken/Malay-sia/ 5744/ 2004 (H5N1)	HA, and HA1	H5 HA-specific IgG in mice	The Immunogen-icity testing of HA1	Pua et al. (2017)
A/Vietnam/1203/04 (H5N1)	HA1	HA-specific antibodies raised against HA1 in mice	The amount of protein levels in seeds was studied.	Ceballo et al. (2017)
A/Indonesia/05/05 (H5N1)	HA1	-	Transient protein expression in *Nicotiana benthami-ana.*	Farsad et al. (2017)
A/Indonesia/05/05 (H5N1)	HA	specific antibodies in mice and ferrets	The effect of c-di-GMP mucosal adjuvant on the elicited immune response.	Major et al. (2015)
A/Chicken/Kurg-an/05/ 2005 (H5N1)	M2	-	Gus reporter was used to record the	Tarasenko et al. (2013)

			amount of expressed protein in *N.benthami-ana.*	
A/Viet Nam/1194/2004 (H5N1)	HA	Specific immune response in mice and chicken	Comparisons between full-length and truncated gene expression were studied.	Mortimer et al. (2012)
A/mallard/Swed-en/ 7206/ 2004 (H7N7)	HA	-	The effect of SEKDEL on protein expression levels was studied.	Kanagarajan et al. (2012)
A/Viet nam/1203/2004 (H5N1)	HA	Neutralizing antibodies in mice	The effect of human or murine Fc fragment fusion heavy chain was tested.	Spitsin et al. (2009)
A/Vietnam/04 (H5N1)	HA, NA	-	The effect of the LicKM	Musiychuk et al. (2007)

	carrier was studied.

1.8. Avian influenza virus in Egypt

The most predominant AIV subtypes in Egypt are HPAI (H5N1 and H5N8) and LPAI (H9N2) (Naguib et al., 2019). The first H9N2 report in Egypt was identified in samples from poultry markets in 2006 by RT-qPCR (Spackman et al., unpublished data) (Nagy et al., 2017), though no virus isolation was reported from chickens. From 2009 to 2012, antibodies against H9 viruses were common in poultry in Egypt (Afifi et al., 2013). The current strain was significantly dissimilar from the first emergence of H9N2 in 2006 after reassortment between H9N2 strains belonging to different genotypes. So far, H9N2 has infected a wide variety of birds in Egypt, including chickens, pigeons, ducks, quails, and turkeys (Arafa et al., 2012a, Naguib et al., 2015, Kandeil et al., 2019, El Sayes et al., 2022). Most outbreaks have been recorded during winter, but outbreaks have been reported throughout the year, especially in the Nile Delta (Arafa et al., 2012b, Abdelwhab and Abdel-Moneim, 2015). Inactivated vaccines using H9N2 strains are commonly utilised in Egypt, and antigenic drift has been recorded in chickens (Kandeil et al., 2014, Adel et al., 2017, Kandeil et al., 2017a, An et al., 2022).

The H5N1 virus was first observed in common teal in Egypt in December 2005 during the sampling of migratory birds. Genetic analysis of this virus revealed a close relationship to the clade 2.2 virus observed months earlier

in migratory birds in China. Clade 2.2 viruses were reported in poultry farms in Egypt from February 2006. Control measures involving the slaughter of 40 million birds over 10 months caused significant losses to the poultry industry. These efforts have not been effective, and the virus has become endemic. Since becoming endemic, the outbreaks of H5N1 in poultry have been mainly seasonal, particularly during the colder months (El-Shesheny et al., 2021).

An AIV surveillance study was initiated in 2009 in Egypt, targeting commercial farms, bird markets, and backyard flocks. Between 2009 and 2010, there was a 5% detection rate of AIV due to H5N1 (Kayali et al., 2011a). Between 2010 and 2013, 10% only of the collected samples were positive and contained H5N1, and the remaining 90% contained H9N2. The H5N1 infection rate declined to 4.6% between 2013 and 2015, and no H5N1 viruses were observed by 2015 (Kayed et al., 2016). Finally, from 2016 to 2018, the detection percentage of H9N2 was 2.9%, with H9N2 being the most frequently isolated virus. No reassortment between the circulating H5N1 and H9N2 viruses has been documented (El-Shesheny et al., 2021).

In 2016, H5N8 viruses were identified in Egypt, possibly introduced from Europe via migratory birds (Kandeil et al., 2017b). The H5N8 virus detection rate has risen, while the H5N1 virus detection rate has decreased drastically. Because H5N8 viruses have not caused human infections, replacement of H5N1 viruses with H5N8 viruses may be helpful from a One Health viewpoint. In 2016, H5N8 displayed higher infection levels, followed by

H9N2 and H5N1. H5N8 was prevalent in Southern Egypt, H9N2 in the Nile Delta, and H5N1 in both regions (El-Shesheny et al., 2021). The recent strains of H5N8 viruses in Egypt are genetically different from the currently used vaccines. Therefore, current poultry vaccines need to be updated to improve their effectiveness against H5N8 outbreaks in Egypt (Salaheldin et al., 2022).

1.9. Aim and objectives

This project aimed to produce a plant-based vaccine against AIVs, particularly for important strains (H5 and H9) in Egypt. This was addressed by the following objectives;

1. Expression of AIV HA proteins in plants. HA protein selection of sequences specific for local Egyptian strains of AIV (H5 and H9) were expressed using a plant-based system and purified as VLPs or HA subunit proteins.

2. Expressed proteins were screened against avian sera to determine whether sera recognised the expressed proteins from birds infected with AIV virus strains of specific subtypes (H5 and H9).

3. Pseudotype virus and ELISA assays were allowed to detect and quantify virus neutralizing and binding antibodies to H5 and H9 strains of AIV.

4. The vaccines were tested for immunogenicity by immunising mice and chickens and measuring elicited immune responses. Antibodies were measured by ELISA and pseudotyped virus assay to determine the ability of

the sera to bind to or neutralize virus infection. Enzyme-linked immunosorbent spot (ELISpot) was used to measure the cytokine-producing T cells in immunised mice.

2.1. Introduction

Plant-based vaccines can be produced by transferring a gene from an organism of interest into a plant for expression to enable heterologous protein expression. Plant vaccine production includes many steps, such as the design of the target antigen, selection of the vector, selection of the plant, method of the transformation of plants, and the gene delivery method. The initial step in vaccine development depends mainly on choosing the protective antigen (or antigens) from the organism of interest and optimisation of gene design.

Gene design usually involves codon optimisation for the plant by removing undesired introns and RNA motifs. Codon optimization is a process in which the gene sequence is changed to reflect the preferred codon usage of the host (plants) to increase expression efficiency (Gustafsson et al., 2004, Webster et al., 2017). The redundancy of the genetic codes facilitates encoding the amino acid using more than one codon, with different preferences occurring in different organisms. Flanking restriction sites can be added to ease the cloning process (Mauro, 2018).

Other potential modifications include additives such as the Kozak sequence that enhance initiation of the translation in 5′-UTR of the gene (Kozak, 1989), a retention signal peptide such as SEKDEL in the 3′-end to retain the gene in the endoplasmic reticulum, and a tag such as 6x Histidine (usually at the

carboxy-terminal of the protein gene) to enable affinity-based purification (Pua et al., 2017).

Second-generation plant expression vectors have resolved low protein yield problems that can carry larger inserts (Gleba et al., 2005). These “deconstructed vectors” include only the target gene and the essential plant virus components necessary for replication, therefore the requirement of *Agrobacterium* to transfer the target gene to the plant cell for expression. A frequently used plant expression vector is the pEAQ-Hyper-Translatable (HT) plasmid based on cowpea mosaic virus (CPMV), which can be used to transfer more than one protein in the same cell, which is ideal for complex VLPs like bluetongue VLPs (Sainsbury and Lomonossoff, 2008, Sainsbury et al., 2009, Mokoena et al., 2019) in which four capsid proteins should be expressed to assemble a VLP. The pEAQ-HT vector backbone has the tomato bushy stunt virus P19 gene to enhance the expression levels and reduce post-transcriptional gene silencing. The pEAQ-HT plant-expression vector allows high-level expression of recombinant proteins in plants, with up to 25% of total soluble protein stated while using *Nicotiana benthamiana* as a host (Sainsbury and Lomonossoff, 2008, Sainsbury et al., 2009).

A transient expression system can produce recombinant subunit vaccines and VLPs in plants. VLPs are nanoparticles made by the assembled viral protein subunits (Nooraei et al., 2021). The VLPs have higher immunogenicity than the recombinant proteins because the epitopes are well presented on the surface of the VLPs for APC attachment and activation

of the immune system. Recombinant proteins need a strong adjuvant or a scaffold system to present to the immune cells to induce a more robust immune response (Stephenson et al., 2003, Levine et al., 2017, Sun et al., 2017). Hepatitis B core antigen (HBcAg) VLP or core-like particles (CLP) can self-assemble into VLPs that are highly immunogenic and extensively used to overcome the recombinant protein poor immunogenicity (Peyret et al., 2020, Zhou et al., 2020). HBcAg consists of 183−185 amino acids that can assemble into a CLP (Cohen and Richmond, 1982, Stahl et al., 1982, Zhou and Standring, 1992, Moradi Vahdat et al., 2021). The CLP is highly flexible, with a large and wide range of epitopes integrated into the system, but this integration may reduce protein immunogenicity (Peyret et al., 2015, Moradi Vahdat et al., 2021). Target epitopes can be presented on the C- or N- terminus or the major immunogenic region of the HBc particle via chemical or genetic integration. Chemical integration is time and cost-consuming due to the need to express and purify the target protein and HBc particles separately and does not allow full control of the gene integration site on the particle (Peacey et al., 2007, Peyret et al., 2020). Genetic integration is limited by the target gene size as it may affect the HBc stability and assembly (Koletzki et al., 1999, Pumpens and Grens, 2001, Lee et al., 2012, Peyret et al., 2020). Prof. David Rowland, University of Leeds, UK, has patented an HBcAg VLP system that allows the recombinant proteins to be presented on its surface; the SUMO (small ubiquitin-related modifier)-tagged recombinant proteins avoid the integration issues. SUMO is a fusion tag with many advantages, such as easing the purification process and

enhancing protein folding (Butt et al., 2005b, Zuo et al., 2005, Marblestone et al., 2006, Chen et al., 2021). The modified HbcAg contains affimers, a high-binding affinity sequence that binds to SUMO-tagged proteins. This system can present multiple antigens to the host for a better immune response (Kingston et al., 2023).

2.2. Material and methods

2.2.1. Phylogenetic analysis of H9N2, H5N1, and H5N8 worldwide

Full-length HA sequences for (H5N1, H5N8, and H9N2) affecting chickens worldwide were obtained from GenBank for phylogenetic analysis. Differences between HA cleavage sites for all the obtained sequences were analysed and compared to our target vaccine strain. The complete list of genes with accession numbers was added in Appendix A. Geneious prime (version 2020.1.2) was used to align the obtained sequences to the consensus vaccine strain using Muscle alignment with the default settings. The vaccine strain was selected after analysing the differences between the recently evolved strain in Egypt. The maximum likelihood tree was generated using Mega X software with a bootstrap of 100 replicates using the default settings for the Jones-Taylor-Thornton model. The clades of H5

were confirmed using the influenza research database (IRD) H5 clades classification tool.

2.2.2. Overview of the cloning strategy

An overview of the design and cloning strategy of the H9(N2) HA gene and H5(N8) (HA) into the expression plasmid pEAQ-HT is shown in Figure 2.1. The description of the construct design and cloning is explained below.

HA gene constructs for H9 and H5 genes were designed using Geneious prime (version 2020.1.2) to contain a 6x Histidine tag (His-tag) at the carboxy (C) terminal of the individual gene to enable affinity-based purification. In addition, restriction sites were incorporated at either terminus: an *AgeI* (New England BioLabs Ltd, UK) restriction site at the 5' end and an *XhoI* (New England BioLabs Ltd, UK) restriction site at the 3' end of the gene sequences were introduced. The target gene was synthesized in the following order; *AgeI* restriction site, Kozak (GCC ACC), a sequence enhanced for efficient initiation sequence of the translation in 5′-UTR of the gene (Kozak, 1989), the signal sequence of tobacco pathogenesis related protein-1a (PR1a) (90 bp (GenBank accession no. X06930) which is responsible for targeting the protein to the ER, replacing the native HA signal sequence, H9N2 or H5N2 HA sequence; followed by a sequence encoding the hexahistidine (6×His) tag for affinity purification, an ER retention signal peptide SEKDEL at the 3′-end to retain the HA in ER, stop codons for stopping translation, and an *XhoI* restriction site (Kanagarajan et al., 2012). The final gene sequences were codon-optimised for expression in *Nicotiana benthamiana* using Geneious

software and synthesized by GeneArt Gene Synthesis (ThermoFisher Scientific). The synthesized cDNA construct was received and cloned into pEAQ-HT (accession number GQ497234) (Appendix I). The His-Tag and SEKDEL were removed using PCR for H9 and H5 VLP expression (Table 2.1).

Table 2.1. Illustration of the constructs used in the study.

Constructs	Codon optimisation	Type of vector	Type of tag	Uses
pEAQ-HT-H9-His-tagged protein	+	pEAQ-HT	Synthesised His-tag at the C terminus of HA	Used in ELISA with post-vaccination sera
pEAQ-HT-H9-VLP	+	pEAQ-HT	No His-tag	Used in ELISA with post-vaccination sera and mice immunisation trial
pEAQ-HT-H5-His-tagged protein	+	pEAQ-HT	Synthesised His-tag at the C terminus of HA	Used in ELISA with post-vaccination sera
pEAQ-HT-H5-VLP	+	pEAQ-HT	No His-tag	Used in ELISA with post-vaccination

				sera, mice, and chicken immunisation trials
pEAQ-HT-H2-His-tagged protein	-	pEAQ-HT	Use vector C-terminus His-tag	-
pHREAC-H9-His-tagged protein	+	pHREAC	Synthesised His-tag at the C- terminus of HA	-
pEAQ-HT-H9-SUMO-tagged protein	+	pEAQ-HT	Use vector C-terminus His-tag	Sent to our collaborators for binding with VelcroVax VLPs
pEAQ-HT-H5-SUMO-tagged protein	+	pEAQ-HT	Use vector C-terminus His-tag	Sent to our collaborators for binding with VelcroVax VLPs

For H2N3, HA was amplified from the H2N3 virus H2N3 (A/mallard duck/England/7277/0), kindly supplied by Prof. Ian Brown (Animal and Plant Health Laboratories, Weybridge) using PCR, and restriction sites *AgeI* and *XmaI* were incorporated at the beginning and the end of the gene to facilitate

the cloning into the pEAQ-HT vector. The BsaI restriction site flanked the $H9N2_{HA}$ for cloning into the pHREAC vector (accession number MK521430) (Appendix J). The pHREAC vector was a gift from Prof. George Lomonossoff (Addgene plasmid #134908; http://n2t.net/addgene:134908; RRID: Addgene_134908).

The TMD was removed from the H9 and H5 genes using PCR to ease the purification process for HBc particle presentation. The genes were designed in the following order; *AgeI* restriction site, Kozak (GCC ACC), a sequence enhanced for efficient initiation sequence of the translation in 5′-UTR of the gene (Kozak, 1989), the signal sequence of tobacco PR1a (90 bp (GenBank accession no. X06930), replacing the native HA signal sequence, H9 or H5 sequence; SUMO tag (Kingston et al., 2023), stop codons for stopping translation, and an *XmaI* restriction site for cloning into the C-terminus with His-Tag of the pEAQ-HT vector.

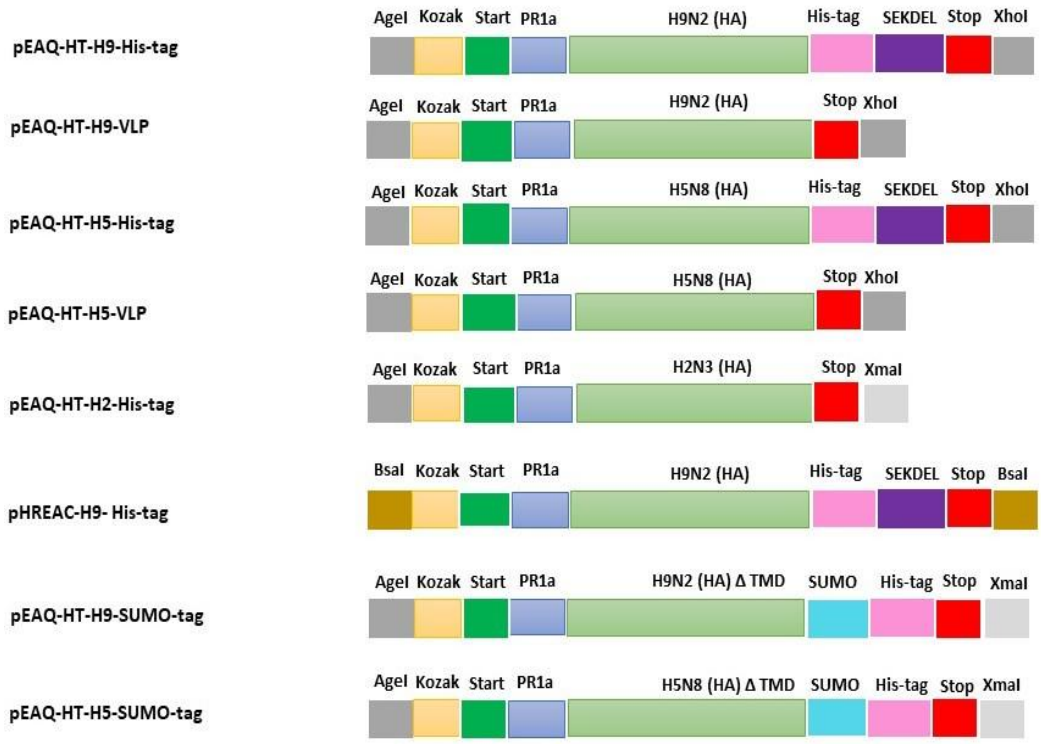


Figure 2.1. Schematic representation of the gene inserts used in this chapter.

Nucleotide sequences for the HA gene from different influenza strains were obtained from (GenBank) according to the recent AIV outbreaks in Egypt and are listed in Table 2.2. The H5 sequence belongs to clade 2.3.4.4 while the H9 belongs to G1 h9.4.2 Western lineage. Complete gene sequences were codon-optimised for expression in *N. benthamiana* and DNA synthesized by GeneArt Gene Synthesis (ThermoFisher Scientific). Final products were supplied as lyophilized preparations cloned into the plasmid, pMA-T. Preparations were reconstituted with 50 µl of nuclease-free water (Qiagen) to a final concentration of 5 µg/ml and stored at -20°C. The HA-

pMA-T construct was transformed into *E. coli* and then restriction digestion for HA was done to anneal the HA into the pEAQ-HT or pHREAC.

Table 2.2. Details about the nucleotide sequences for the HA gene from different influenza serotypes were obtained from GenBank.

Influenza virus serotype	**GenBank accession number**	**Country of origin**	**Year isolated**	**Species isolated from**	**nucleotide length (bp)**
$H9N2_{HA}$	KY910830	Egypt	2016	chicken	1683
$H5N8_{HA}$	MH986133	Egypt	2018	chicken	1717

2.2.3. End-point Polymerase Chain Reaction

Avian influenza A/mallard duck/England/7277/06 (H2N3) was cultivated in allantoic fluid of embryonated hens' eggs and RNA was extracted from the allantoic fluid using QIAamp viral RNA purification kit (Qiagen, UK).

H2N3 cDNA was generated from 1 µg H2N3 viral RNA using UltraScript 2.0 cDNA synthesis kit (PCR Biosystems) containing a mix of oligo (dT) and random hexamers.

The reactions were prepared to a final total volume of 20 µl, detailed below.

5x cDNA Synthesis Mix	4 µl
UltraScript 2.0 for cDNA Synthesis	1 µl
RNA	5 µl
Water	10 µl

The reaction mix was incubated at 50–55°C for 10–30 minutes and then at 95°C for 10 minutes.

$H2N3_{HA}$ cDNA was amplified using MyFi™ DNA Polymerase (Bioline) using H2N3 HA1 primers and annealing temperatures of 59°C and 60°C (Table 2.3).

The synthesised H9 and H5 genes were multiplied using MyFi™ DNA Polymerase with H9/H5 HA primers for removing the His-tag with annealing temperatures 57°C−63°C (Table 2.3).

The H9 gene was amplified using H9pH primers for cloning into pHREAC vector with annealing temperatures of 59°C and 60°C (Table 2.3).

The synthesised H9 and H5 genes were multiplied using MyFi™ DNA Polymerase with (H9-SUMO) and (H5-SUMO) primers for removing the TMD with annealing temperatures of 58°C and 59°C (Table 2.3). The SUMO template was provided by our collaborators and amplified using SUMO primers to add the restriction sites to ease the cloning with the H9 and H5 genes into the pEAQ-HT vector with annealing temperatures of 59.5°C and 60.4°C (Table 2.3).

All PCR reactions were prepared to a total volume of 25 µl, as detailed below;

5x MyFi Reaction Buffer	5 µl
Primers 10 µM each	1 µl
MyFi DNA Polymerase	1 µl
Template	3 µl

Nuclease-free water 15 µl

The following thermal profile was used to run the gradient PCR on a thermocycler (Gene Touch Plus Digital Gradient DNA PCR Machine Thermal Cycler (Hangzhou Bioer) Technology, China).

Initial denaturation	95°C	1 minute	
Denaturation	95°C	30 seconds	35 cycles
Annealing	differs according to the primer		
Extension	72°C	90 seconds	

After the PCR reaction, the sample was mixed with 5X loading buffer and loaded directly onto a 1 % agarose gel. The band was extracted and purified using Illustra Ligation (GE Healthcare) gel purification kit according to manufacturer guidelines. The BK79 and BK80 primers were used for sequencing the amplified inserts into the pEAQ-HT vector using Source BioScience Sanger sequencing.

Table 2.3. Primer sequences used in PCR for the preparation of expression constructs.

Primer name	**Sequence (5′‘→3′‘)**	**Function**	**Annealing temperature**
H2N3 (HA1)	**Forward**: GCAACCGGTAACAATGGCCATC	It was used to amplify the HA	59°C and 60°C

	Reverse: GATCCCGGGAATGCAGATCC TGC	gene using the H2N3 virus.	
BK 79	GGTTCTATAAGAAATCTAG	Sequencing primers were used to confirm the insert sequence in the pEAQ-HT plasmid.	−
BK 80	CTGCTAAACAGGAGCTCAC		
H9/H5 (HA)	**Forward:** GCAACCGGTAACATGGGCTT CGTGCTGTTCTCTCAGCTGCC TTCTTTCCTTCTTGTGTCT	It was used to amplify the H9N2 and H5N8 (HA) genes without His-tag.	57°C−63°C
	Reverse: GATCTCGAGCTA AATGCAGATATTGCACCGGCA GCTT		
H9pH	**Forward:** GCA GGTCTCGAAAATTAAAA ATGGGCTTCGTGCTGTTC	It was used to insert the *BsaI* restriction sites into the H9 gene for cloning with pHREAC	59°C and 60°C
	Reverse: CAGGGTCTCGAGCGTTAAAG CTCGTCCTTCTCGCT		

H9-SUMO	**Forward:** GCAACCGGTAACA ATGGGCTTCGTGCTG	It was used for removing the TMD from the H9 and H5 genes	58°C and 59°C
	Reverse: TGCCTCGAGGGTCAGGATCTT GTAGGTGCC		
H5-SUMO	**Forward:** GCAACCGGTAACA ATGGGCTTCGTGCTG		
	Reverse: TGCCTCGAGGCTCAGAATCTG GTAGGTGCC		
SUMO primers	**Forward:** GCACTCGAGATGTCGGACTC AGAAGTCA	It was used for adding the restriction sites to ease the cloning with the H9 and H5 genes into the pEAQ-HT vector	59.5°C and 60.4°C
	Reverse: TATCCCGGGGCTGCCGCGCG GCACCAGACC		

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) was used to visualise the DNA. AGE steps were as follows; firstly, agarose was dissolved in 1X TAE buffer to 1% (w/v) by heating in a microwave. Secondly, 3 µl of Nancy–520 (Sigma) was added to each slightly cool agarose solution, and the solution was poured

into a gel tray, with an appropriate comb added, and allowed to solidify at room temperature. Then, samples were prepared by mixing 7 µl of the individual sample with 2 µl of 5X loading dye (Bioline) before loading into individual wells. A 10 kb DNA marker was loaded in a single lane (Bioline) for each gel. Finally, gels were run for 1 hour at 115 V submerged in 1X TAE buffer in a gel electrophoresis tank (Power Pac Basic TM, Bio-Rad, Segrate, Italy). DNA bands were visualized using a UV transilluminator.

2.2.5. Cloning of the HA gene into the pEAQ-HT and pHREAC vectors

The commonly used plant expression vectors, namely pEAQ-HT and pHREAC, were compared in this study. The expression vector, pEAQ-HT, was provided by Prof. George Lomonossoff at the John Innes Centre (Sainsbury et al., 2009, Peyret and Lomonossoff, 2013). For the cloning of H9, H5, and H2 genes into pEAQ-HT, the plasmid was digested with *AgeI* and *XhoI* (New England BioLabs Ltd, UK) restriction enzymes to excise the respective synthetic genes from the carrier vector (pMA-T) in case of H9 and H5 genes, and PCR-amplified HA in case of H2 gene. Each gene was subsequently inserted into pEAQ-HT, and digested with *AgeI* and *Xho*I, using T4 DNA Ligase (New England BioLabs) according to the instructions of the manufacturer.

The pHREAC vector was used to compare the protein expression levels with the pEAQ-HT. The pHREAC vector was a gift from Prof. George Lomonossoff (Addgene plasmid # 134908; http://n2t.net/addgene:134908; RRID: Addgene_134908). Golden gate cloning was used for cloning the H9

gene into the pHREAC vector using the Golden Gate Assembly kit (BsaI-HF® v2, New England BioLabs) according to the instructions of the manufacturer.

2.2.6. Preparation of *E. coli* and *Agrobacterium* competent cells

Escherichia coli (*E. coli*) Subcloning Efficiency™ DH5α Competent Cells (ThermoFisher Scientific, UK) were used as host cells for pEAQ-HT plasmids. *E. coli* was transformed using a Mix&Go transformation kit (Zymo Research). *E. coli* culture was grown into LB broth overnight at 37°C, and 0.5 ml of fresh culture was added to 50 ml of Zymo broth™ in a 500 ml culture flask. The culture was shaken vigorously (150–250 rpm) at 17–22°C until OD_{600nm} was 0.4–0.6. The culture was transferred into ice for 10 minutes and then centrifuged (Heraeus™ Pico™ 17 Microcentrifuge, Thermo Scientific®) at 3500 rpm at 4°C. The supernatant was discarded, and the cells were resuspended into 5 ml ice-cold 1x wash buffer and then centrifuged again. The supernatant was discarded, and the cells were resuspended into a 5 ml ice-cold 1x competent buffer. Aliquots (0.2 ml) of cell suspension were distributed into microcentrifuge tubes and stored at -80°C.

A. tumefaciens GV3101 strain was kindly provided by Dr Kostya Kanyuka (Rothamsted Research, UK). GV3101 culture was grown into 5m LB broth containing 15 µg/ml rifampicin overnight at 28°C with vigorous agitation for 22 hours, and 5 ml of fresh culture was added to 500 ml of LB containing 15 µg/ml rifampicin in a 500 ml culture flask. The culture was shaken vigorously

at 28°C until OD_{600nm} was 0.5. The culture was transferred into ice for all further steps and then centrifuged (Heraeus™ Pico™ 17 Microcentrifuge, Thermo Scientific®) at 4000 *g* for 10 minutes at 4°C. The supernatant was discarded, and the cells were resuspended into 5 ml ice-cold water and then centrifuged again. The supernatant was discarded, and the cells were resuspended into a 5 ml 10% (v/v) ice-cold, sterile glycerol. Aliquots (50 µl) of cell suspension were distributed into microcentrifuge tubes and stored at -80°C.

2.2.7. Transformation of competent *E. coli* with the constructed plasmids

E. coli were used as host cells for pEAQ-HT plasmids. Plasmids were transformed into competent *E. coli*. The bacterial transformation was performed according to the published protocol (Addgene, 2017). Briefly, the competent cells were thawed on ice for 30 minutes. Plasmids (5 µl) were mixed into 50 µl of competent cells in microcentrifuge tubes and incubated on ice for 30 minutes. The mixture was heat shocked in an incubator at 42°C for 60 seconds and put back on the ice for 2 minutes. SOC media without antibiotics (750 µl) was added to the mixture and were grown in a shaking incubator (Thermo Forma 420 Incubator Shaker, Thermo Scientific®) at 37°C for 45 minutes. Transformed samples were then plated on a Petri dish of LB agar containing 50 µg/ml kanamycin for pEAQ-HT and pHREAC vector or 100 µg/ml ampicillin for pMA-T vector for positive selection and positioned face down in a 37°C incubator overnight. Positive colonies were selected

and added to 4 ml of liquid LB broth containing the suitable antibiotic and incubated overnight on a shaker (225 rpm) at 37°C, and the plasmids were purified using plasmid miniprep according to the instructions of the manufacturer.

The pEAQ-HT vector plasmids were digested using *AgeI* and *XhoI* (New England BioLabs Ltd, UK) restriction enzymes. Then the digested product was run on gel electrophoresis to confirm the size of the ligated genes. Plasmids were verified via Sanger DNA sequencing (Source Bioscience, UK).

2.3. Results

2.3.1. Phylogenetic analysis

A worldwide phylogenetic analysis of influenza A virus (IAV) (H5) in chickens was performed. According to the analysis of the tree in chicken, the clades originated from American non-goose Guangdong (Am-non GsGD). The clades were classified into Eurasian non-goose Guangdong (EU-non GSGD), clade 7.2, clades (1, 1.1, 1.1.2), clades (2.1.3, 2.1.3.2, 2.1.3.2a, 2.1.3.3), clades 2.1.1, clade 2.5, clades (2.2, 2.2.1, 2.2.1.1, 2.2.1.1a, 2.2.2.1, 2.2.2), clades (2.3.2.1, 2.3.2.1 like 2.3.2.1a, 2.3.2.1c), clades (2.3.4, 2.3.4.1, 2.3.4.3, and 2.3.4.4). H5N1 first originated in Egypt under the clade 2.2.1. After point mutations occurred in H5N1, the 2.2.1.1a clade was introduced in Egypt, and both clades in Egypt were genetically related to H5N1 sequences in Palestine and Israel. All $H5N8_{HA}$ sequences in Egypt were grouped under 2.3.4.4 clades. The strain used for the plant-based vaccine

against $H5N8_{HA}$ in Egypt (marked in blue) belongs to clade 2.3.4.4 (Appendix E). The selected H5N8 strain A/chicken/Egypt/FL6/2018 was the recent circulating strain causing outbreaks in Egypt.

Phylogenetic analysis of IAV H9N2 was recorded in chickens worldwide. According to the tree analysis in chickens, all H9N2 strains were grouped under G1 h9.4.2 (Western lineage), which is currently widely circulating worldwide. The selected H9N2 strain A/Egypt/ZU63/2016 was the recent circulating strain causing outbreaks in Egypt. The strain used for the plant-based vaccine against $H9N2_{HA}$ in Egypt (marked in blue) belongs to the G1 h9.4.2 Western lineage (Appendix F).

2.3.2. Cleavage site analysis

Full-length HA sequences (H5N1, H5N8, and H9N2) affecting chicken worldwide were collected for cleavage site analysis. Cleavage sites for all the sequences were analysed and compared to our target vaccine strain. The primary determinant of the HPAIV H5 is the multi-basic cleavage site (MBCS) in poultry. The H5N1 strain virus was predominant in Egypt until 2016. The H5N1 cleavage site sequence is mostly KRRKKR↓ , while sometimes the lysine (K) is replaced by arginine (R), a basic amino acid. In 2016, HPAIV H5N8 started to appear in Egypt and mainly has RKRRKR↓ and RRRKKR↓ (which is our target vaccine strain) at the cleavage site (Appendix G). MBCS is the primary key of pathogenicity in H5N1 HPAIV and

is cleaved by a furin-like protease, abundant in chicken tissues resulting in severe infection.

H9N2 LPAIV has a dibasic cleavage site with a PARSSR↓ motif. H9N2 viruses in Egypt belong to G1 or western lineage, which is endemic in Eurasia and the Middle East (Appendix H).

2.3.3. Preparation of the expression constructs

The pEAQ-HT-H9-His-tagged plasmids were confirmed using restriction digestion with *AgeI* and *XhoI* using gel electrophoresis and sequencing. The bands were visible at the expected size of around 1.7 kb for the H9 gene (Figure 2.2).

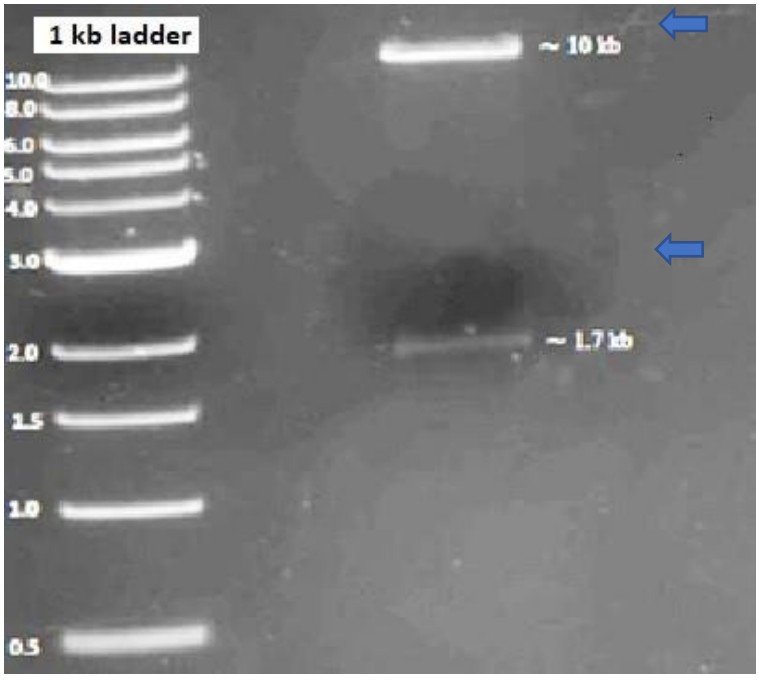


Figure 2.2. Agarose gel electrophoresis of the digested pEAQ-HT-H9-His-tagged plasmids. The pEAQ-HT vector size is around 10 kb and the HA insert size is approximately 1.7 kb, as represented by arrows.

The pEAQ-HT-H5-His-tagged plasmids were confirmed using restriction digestion with *AgeI* and *XhoI* using gel electrophoresis and sequencing. The bands were visible at the expected size of around 1.8 kb for the H9 gene (Figure 2.3).

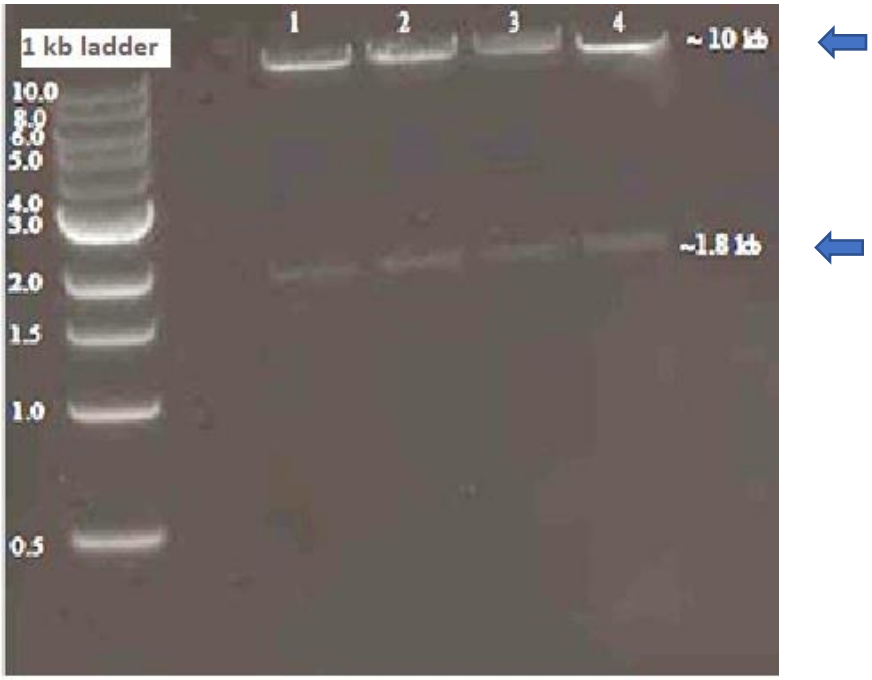


Figure 2.3. Agarose gel electrophoresis of the digested pEAQ-HT-H5-His-tagged plasmids from four independent minipreps. The pEAQ-HT vector size is around 10 kb, and the HA insert size is around 1.8 kb, as represented with arrows.

The full pEAQ-HT-GFP plasmid, kindly provided by Prof. George Lomonossoff at the John Innes Centre, was confirmed using restriction digestion with *AgeI* and *XhoI* using gel electrophoresis (Sainsbury et al., 2009). The bands were visible at the expected size of around 740 bp for the H9 gene (Figure 2.4).

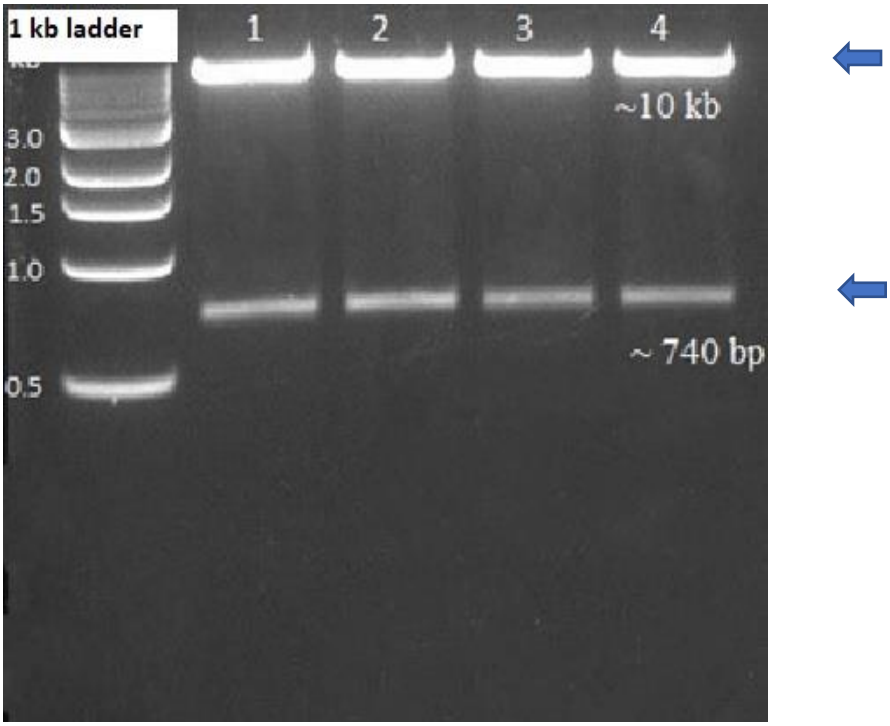


Figure 2.4. Agarose gel electrophoresis of the digested pEAQ-HT-GFP vector from four independent minipreps. The pEAQ-HT vector size is around 10 kb, and the GFP size is around 740 bp, as represented with arrows.

H2N3$_{HA}$ was amplified using PCR and then analysed using gel electrophoresis. The H2 gene size is around 1.7 kb (Figure 2.5).

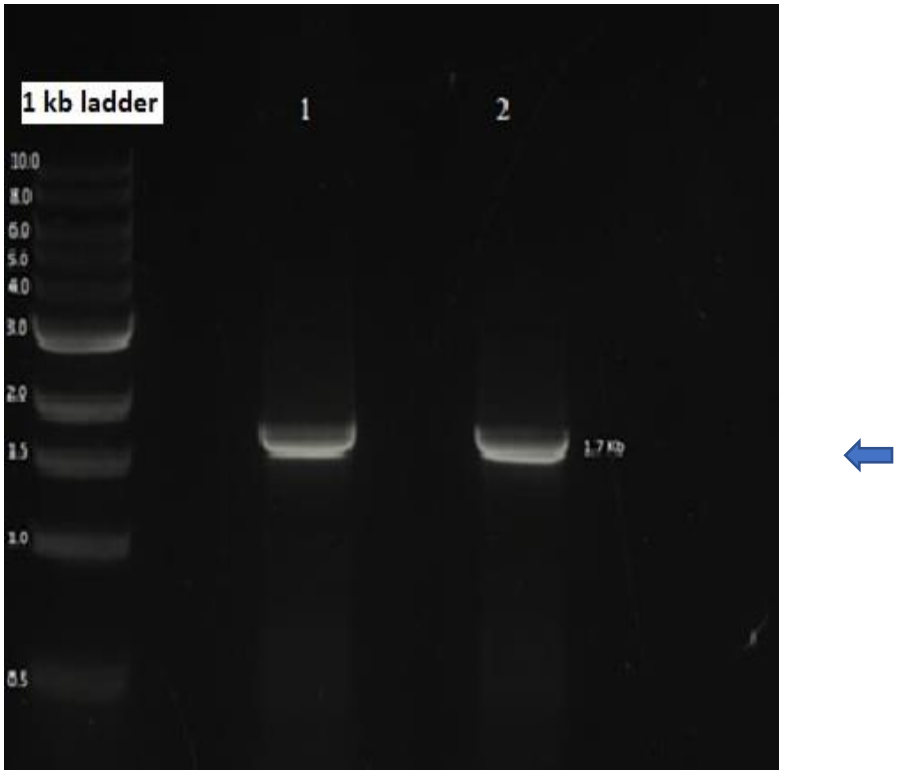


Figure 2.5. Agarose gel electrophoresis of the H2 PCR-amplified gene with 59°C and 60°C annealing temperatures, respectively.

The cloning of H2 into the pEAQ-HT plasmid was confirmed using colony PCR with HA1 primers and then analysed using gel electrophoresis. The H2 gene size is around 1.7 kb, as represented by the arrow (Figure 2.6).

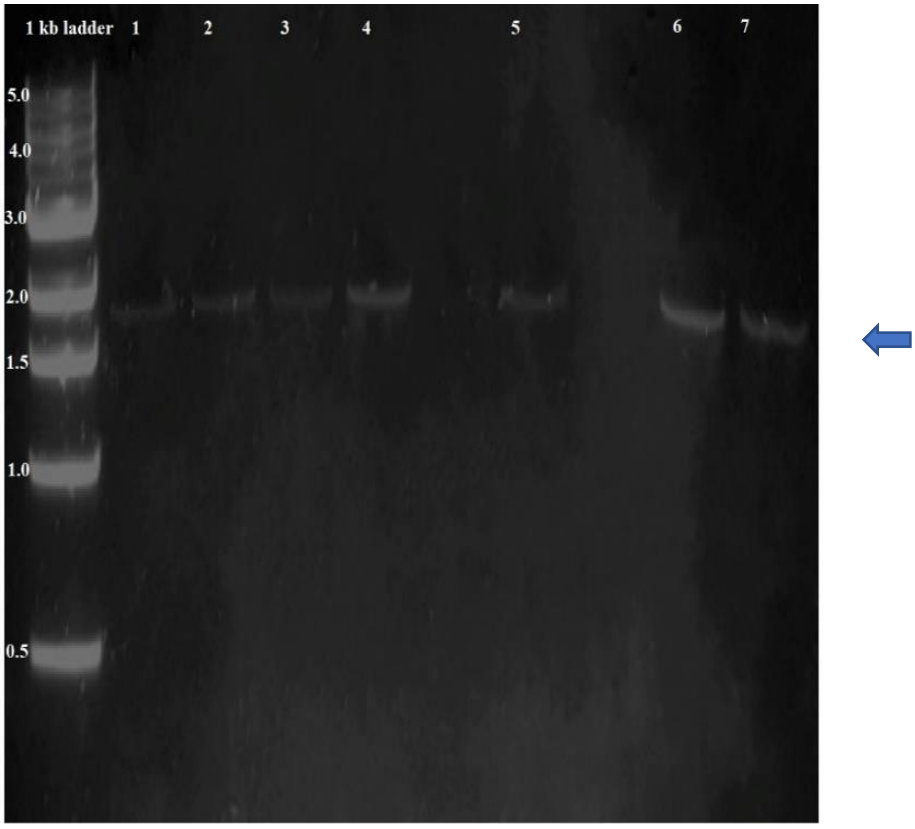


Figure 2.6. Agarose gel electrophoresis of the cloning PCR of pEAQ-HT-H2-His-tagged plasmids. Lanes 1−4 represent HA PCR product from plasmids generated using a 1:3 ligation ratio, while lanes 5−7 represent a 1:5 ligation ratio. The arrow represents the H2 gene size of around 1.7 kb.

H9 was amplified using PCR to delete the His-tag and then analysed using agarose gel electrophoresis. The product size is around 1.7 kb (Figure 2.7).

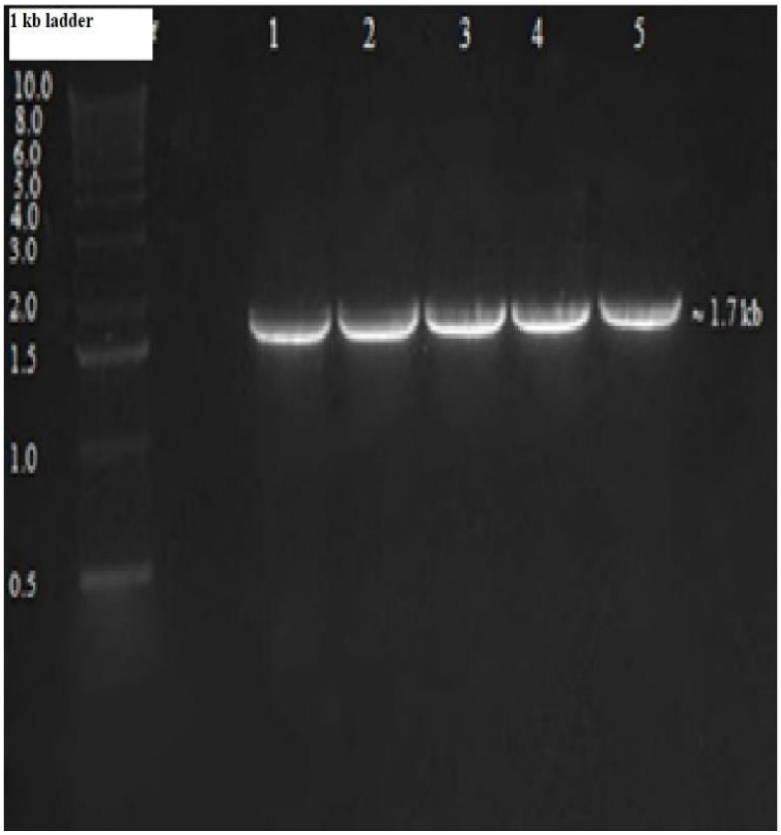


Figure 2.7. Demonstrating the H9 PCR-amplified gene using agarose gel electrophoresis with annealing temperatures 57, 59, 60, 62, and 63°C, respectively. The arrow represents the H9 at the expected size of around 1.7 kb.

H5 was amplified using PCR to delete the His-tag and then analysed using agarose gel electrophoresis. The product size is around 1.8 kb (Figure 2.8).

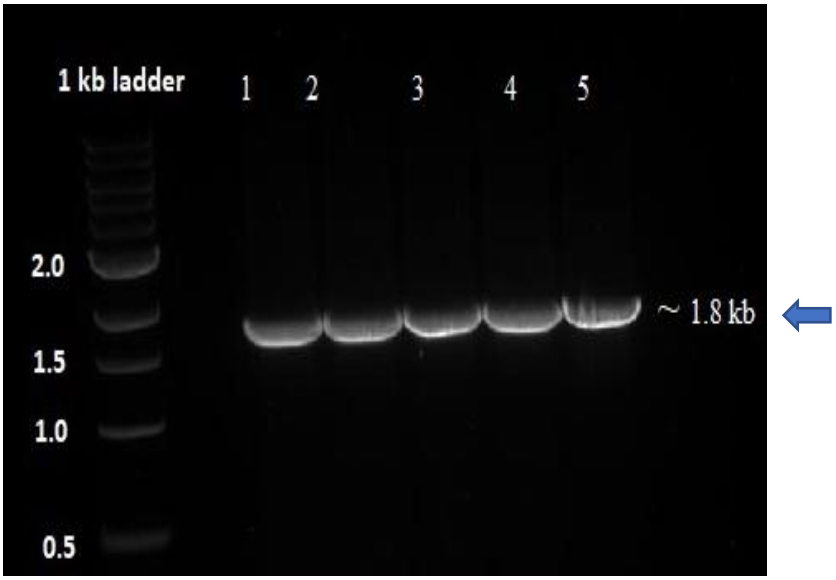


Figure 2.8. Demonstrating the H5 PCR-amplified gene using agarose gel electrophoresis with annealing temperatures 57, 59, 60, 62, and 63°C, respectively. The arrow represents the H5 at the expected size of around 1.8 kb.

The H9 gene was amplified using H9pH primers to add the BsaI sites for cloning into the pHREAC vector with annealing temperatures of 59°C and 60°C (Figure 2.9).

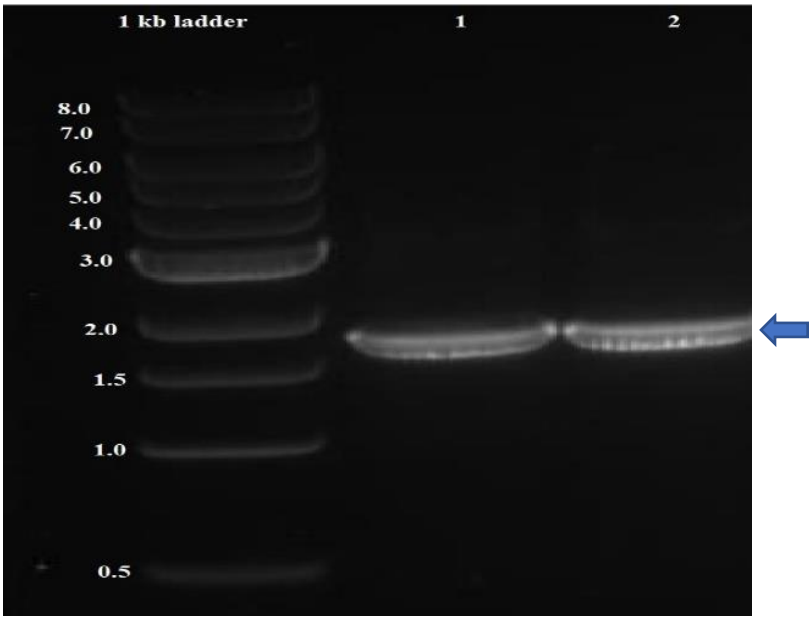


Figure 2.9. Agarose gel electrophoresis of H9 PCR-amplified with H9 Ph primers with 59°C and 60°C annealing temperatures, respectively. The arrow represents the H9 at the expected size of around 1.7 kb.

H9 and H5 genes were amplified using PCR to delete the TMD and then analysed using agarose gel electrophoresis. The product size is around 1.6 and 1.7kb for H9 and H5, respectively (Figure 2.10).

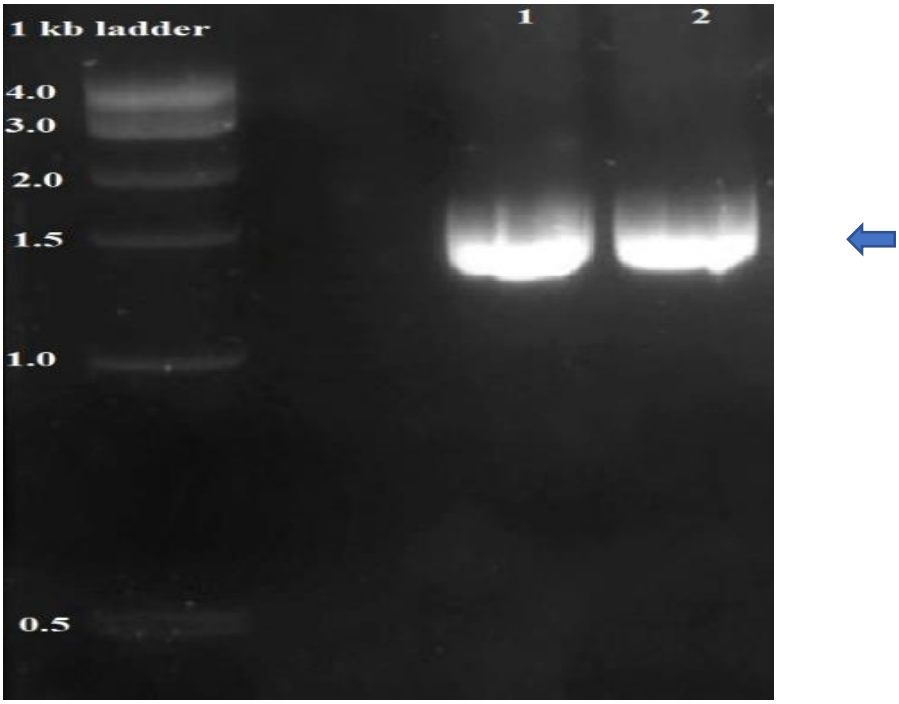


Figure 2.10. Demonstrating the H9 and H5 PCR-amplified Δ TMD using agarose gel electrophoresis. Lane 1 represents H9 and lane 2 represents the H5 gene. The arrow represents the size of H9 and H5, around 1.6 and 1.7 kb, respectively.

SUMO tag was amplified using PCR and then analysed using agarose gel electrophoresis. The product size is around 333 bp (Figure 2.11).

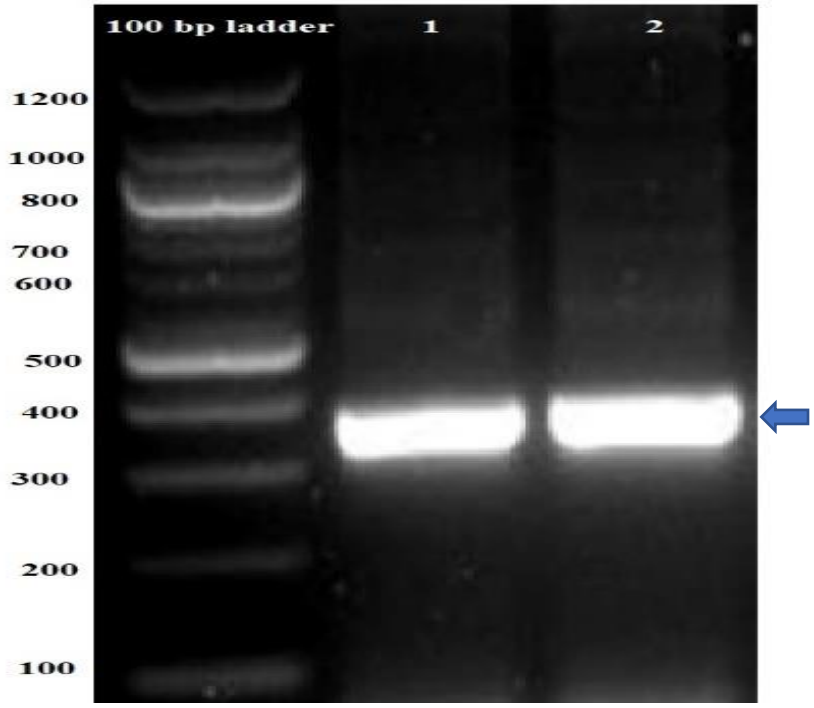


Figure 2.11. Demonstrating the PCR-amplified SUMO gene samples using agarose gel electrophoresis with annealing temperatures of 59.6 and 60°C, respectively. The arrows represent the size of the SUMO-tag of around 333 bp.

Cloning of pEAQ-HT-H9-SUMO-tagged and pEAQ-HT-H5-SUMO-tagged plasmids was confirmed using H9 and H5 SUMO primers and analysed using agarose gel electrophoresis. SUMO insert size was 333 bp and H9 and H5 inserts were around 1.6 kb (Figure 2.12).

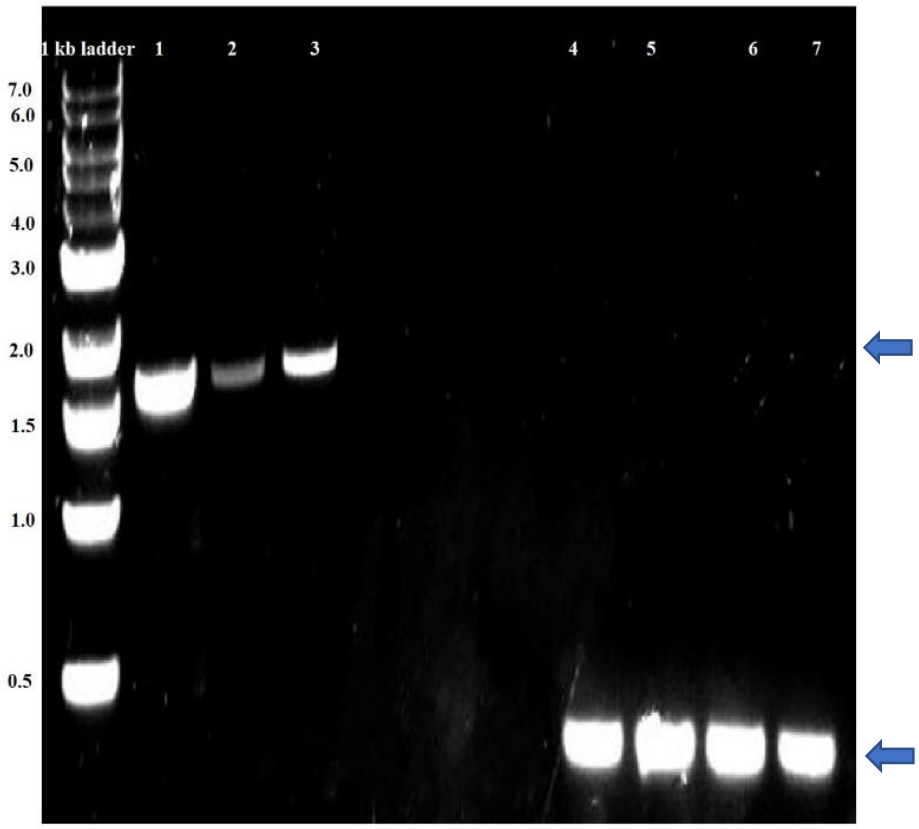


Figure 2.12. Agarose gel electrophoresis of the cloning PCR of pEAQ-HT-H9-SUMO-tagged and pEAQ-HT-H5-SUMO-tagged plasmids. Lanes 1,2 represent the H9 template, lane 3 is the H5 template amplified with H9 and H5 primers with a product size of around 1.6 kDa, lanes 4,5 are H9 template, and lanes 6,7 are the H5 template amplified with SUMO primers with a product size of around 333 bp and all represented by arrows.

2.4. Discussion

2.4.1. Phylogenetic analysis of H5 tree worldwide

The phylogenetic analysis of the H9 tree showed the relationship between H9 serotypes worldwide. Following the first isolation of H5 HPAIV in Scotland (Pereira et al., 1965), the causes of other HPAIV epidemics were

identified and subtyped. The HPAIV strains, currently enzootic in poultry worldwide, are derived from the H5N1 HPAIV A/goose/Guangdong/1/96 (Gs/Gd), demonstrated in the phylogenetic tree. In 1996, the Gs/Gd-H5 HPAIVs were first reported in geese (Xu et al., 1999) and became enzootic in Southeast Asia and China.

They spread to Europe, Africa, and North America between 2003 and 2004, most likely by migratory birds (Chen et al., 2005, Verhagen et al., 2021).

Clade 2 was the most effective since it repeatedly reinfected wild birds (Chen et al., 2005, Liu et al., 2005, Hill et al., 2022). it was able to spread from its source in China to Europe, Asia, America, and Africa (Chen et al., 2006, Cattoli et al., 2009, Lee et al., 2015, Lycett et al., 2020). Since 2014, H5 outbreaks in wild birds have increased, and this rise is mainly attributable to the introduction of H5 HA lineage 2.3.4.4 with different NA strains, including H5N8, H5N6, and H5N2 (Li et al., 2021). Since 2016, Clade 2.3.4.4.b H5N8, which originated in China, has caused repeated outbreaks in wild birds and showed high virulence in ducks (Leyson et al., 2021, Scheibner et al., 2022). Moreover, a reassortant H5N1 strain developed from clade 2.3.4.4b has almost substituted clade 2.3.4.4.b H5N8. This has led to increased outbreaks in wild birds and poultry since November 2021 (Pyankova et al., 2021, Wille and Barr, 2022, Xie et al., 2022).

2.4.2. Phylogenetic analysis of the Egyptian H5 strains

In December 2005, during migratory bird sampling throughout the Damietta Governorate in Egypt, the H5N1 virus was detected for the first time in Egypt

in the common teal. A/bar-headed goose/Qinghai/65/2005 clade 2.2 viruses, detected earlier after the major mortality of migrating birds in Qinghai Lake, China, was closely related to this virus according to genetic analysis. After a decade, Clade 2.2.1 was introduced in both vaccinated and non-vaccinated birds in Egypt and replaced clade 2.2. Clade 2.2.1 viruses were divided into clades 2.2.1.1 and 2.2.1.1a. For unknown reasons, clade 2.2.1.1a disappeared in 2014. Clade 2.2.1.2. was introduced due to a drift in clade 2.2.1.1 (Kayali et al., 2011b, Abdelwhab et al., 2012, El-Shesheny et al., 2014, Arafa et al., 2015, Abdelwhab et al., 2016, Kayali et al., 2016). H5 HPAI Viruses in Egypt were distributed into three groups: A, B, and C. Group A (clade 2.2.1) included isolates from 2006 to 2007, Group B (clade 2.2.1.1 and clade 2.2.1.1a) included isolates from 2008 to 2010. Group C (clade 2.2.1.2) included isolates from 2011 to 2012 (El-Shesheny et al., 2012, El-Shesheny et al., 2021), as demonstrated in the H5 tree.

Most Gs/GD lineage H5 viruses have been identified with the N1 NA for over ten years. However, the identification and spread of H5N8 viruses in China in 2010 caused a change in this tendency. Initial reports of H5N8 viruses were in wild birds and poultry in China. These viruses spread to Europe and the US via the migration of wild birds (Harder et al., 2015, Lee et al., 2017). Another wave of the H5N8 subtype spread from Asia occurred in 2016 (Beerens et al., 2017, Fusaro et al., 2017, Kleyheeg et al., 2017, Pohlmann et al., 2017, Scoizec et al., 2018). These HPAI H5N8 viruses were detected in Egypt in November 2016, probably from Europe belonging to clade 2.3.4.4

as shown in the H5 tree (Kandeil et al., 2017b). Detections of the H5N8 virus have risen, whereas those of the H5N1 virus have sharply declined. Replacing H5N1 viruses with H5N8 viruses may be advantageous because H5N8 viruses have not yet shown any zoonotic importance (El-Shesheny et al., 2021).

2.4.3. Cleavage site analysis of worldwide H5 HPAIV

Since AIV genomes do not encode proteases, host proteases are responsible for cleaving HA0. One basic amino acid, an arginine (R) or, in certain circumstances, a lysine (K), is present at the cleavage site of LPAIVs. Arginine (Arg or R) and lysine (Lys or K), two basic amino acids, are crucial for the function of cleavage sites. Trypsin-like serine proteases expressed in particular tissues can detect this monobasic cleavage site. The respiratory tract and gastrointestinal tract of chickens express trypsin-like proteases, which restrict the LPAIV spread to these organs. In contrast, proteases from the subtilisin-like proprotein convertase family, such as furin, cleave the MBCS from HPAIVs, which include a minimum of four basic amino acids (Vey et al., 1992, Kido et al., 2012).

The widespread expression of this family of proteases in chickens enables systemic viral spread, which results in serious infection attributed to HPAIV infections (Chen et al., 1998, Luczo et al., 2015).

RXR/KR is the minimum consensus sequence of the AIV cleaving furin proteases, and it is believed that the amino acid sequence up to position six (P6) to the C-terminus of HA1 influences furin cleavage (Horimoto and

Kawaoka, 1997, Neumann et al., 1999). Mutations in the arginine on the P1 to P4 positions in the cleavage site decreased cleavability (Horimoto et al., 1995a, Tian et al., 2005).

The structure of the cleavage site motif modifies the activating host cell proteases facilitating cleavage (Chen et al., 1998). The presence of a PBCS motif is correlated with increased pathogenicity of AIV. The minimum consensus sequence at the cleavage site when Lysine is present at position P4 is KK/RK/R/XR↓ (Kido et al., 2012). The typical H5 LPAI HACS motif has Arginine at P1 and P4: RETR↓ (Swayne, 2009). In rare cases, the Arg is changed to Lys. The consensus motif of the HPAI H5 HACS is RXR/KR↓ (Stieneke-Gröber et al., 1992, Vey et al., 1992). H5N1 cleavage site sequence is mostly KRRKKR↓, while sometimes the Lysine (k) is replaced by Arginine (R). In 2016, HPAIV H5N8 started to appear in Egypt and mainly has RKRRKR↓ and RRRKKR↓ (which is our target vaccine strain) at the cleavage site, which is an HPAI MBCS. Both H5N1 and H5N8 showed continued mutations at the cleavage site, causing sudden death of chicken and wide infection spread. Additionally, Egypt is using an imported vaccine from China which is different from the field strain, which illustrates why Egypt can not stop the attacks of AIV every year (El-Shesheny et al., 2023).

2.4.4. Phylogenetic analysis of H9 strains worldwide

The phylogenetic analysis of the H9 tree showed the relationship between H9 serotypes worldwide. In the US state of Wisconsin in 1966, H9N2 viruses were first detected in turkeys (Homme and Easterday, 1970). The virus was

sporadically isolated during the following decades during outbreaks in poultry in the United States, as well as from wild birds and ducks across Europe and Asia (Shortridge, 1992). The virus was initially detected in chickens in China in the early 1990s and then spread over most of Africa and Asia (Guan et al., 1999).

The H9 tree showed that the H9N2 viruses are divided into two major clades, the Eurasian clade and the American clade. American clade is found in wild birds and turkeys. Eurasian lineage is subdivided into three clades originating from A/quail/Hong Kong/G1/1997, which is known as G1, A/chicken/Beijing/1/94, and A/chicken/Hong Kong/Y439/1997, which is known as BJ94 (also named as the G9 or Y280 lineage) and Y439 (also named as the Korean lineage) lineages (Guo et al., 2000). The G1 lineage is subdivided into Eastern and Western sublineages (Dong et al., 2011, Fusaro et al., 2011, Pu et al., 2015, Nagy et al., 2017). Viruses of the BJ94 lineage can be detected in China, Cambodia, Vietnam, and Indonesia, as demonstrated in the H9 tree. Eastern or G1 lineage is also detected in Vietnam, China, and Cambodia, mainly affecting quails and chickens, as shown in the H9 tree. Y439 lineage is detected in wild birds with a sublineage endemic in South Korea and spread over Europe and Asia (Peacock et al., 2019).

The H9N2 virus is thought to be widespread in Pakistan and Bangladesh, as well as maybe in some parts of Afghanistan, India, and Nepal (Cameron et al., 2000, Alexander, 2007, Tosh et al., 2008, Brown, 2010, Negovetich et al., 2011, Hosseini et al., 2017). Most viruses detected in South Asian poultry

belong to G1 "Western" lineage, with a few Y439 viruses from migratory wild birds. Most of the H9N2 viruses detected in the Middle East are of the G1 "Western" sublineage, with a few Y439 viruses from migratory wild birds. In the Middle East, including Egypt, Saudi Arabia, Israel, Iran, and the United Arab Emirates, H9N2 is endemic in poultry (Cameron et al., 2000, Alexander, 2003, Aamir et al., 2007, Alexander, 2007, Banet-Noach et al., 2007, Monne et al., 2013, Nagy et al., 2017). Additionally, the virus has frequently been detected in Jordan, Oman, Lebanon, Kuwait, and Iraq (Barbour et al., 2006, Monne et al., 2007, Brown, 2010, Body et al., 2015, Kraidi et al., 2017) as shown in the H9 tree.

2.4.5. The phylogenetic and cleavage site analysis of Egyptian H9 strains in relation to the worldwide H9 strains

H9N2 viruses were endemic in many African countries such as Egypt, Tunisia, and Libya (Alexander, 2007, Tombari et al., 2011, Monne et al., 2013, Kammon et al., 2015). Moreover, since 2016, the virus has been detected for the first time in West and North Africa, including Ghana, Burkina Faso, and Morocco (El Houadfi et al., 2016, Zecchin et al., 2017, Awuni et al., 2019). G1 ‘Western’ sub-lineage detected in Africa is genetically related to the one circulating in the Middle East, including Egypt (Peacock et al., 2019).

Proteolytic cleavage of the HA is a vital step in the virulence of IAVs (White et al., 1981). The HA0 precursor must be split into the HA1 and HA2 subunits for infection to take place, revealing a fusion peptide for membrane fusion.

H9N2 virus infection is limited to the respiratory and intestinal tracts due to the protease activity (Sun et al., 2013, Tse et al., 2014, Li et al., 2017, Pu et al., 2017). H9N2 viruses differed in the cleavage site amino acid and were divided into monobasic, dibasic, and tribasic. Viruses with dibasic PAKSSR, PSRSSR/G-motif, and PARSSR/G-motif and tribasic PAKSKR/G-motif belong to G1 and Y280 sublineages that are endemic in poultry in the Middle East including Egypt and Asia and have zoonotic importance. The tribasic cleavage site elevated the virus virulence, stability, and spread in chickens. The North American lineage and Y439 sublineage usually include the monobasic H9N2 viruses that contain one basic amino acid at the P1 cleavage site residue without any zoonotic hazard, which are prevalent mainly in Cambodia and Vietnam (Zhang et al., 2021).

2.4.6. Cloning of the constructs into *E. coli*

In this chapter, H9 and H5 were codon-optimised and synthesised for cloning into pEAQ-HT or pHREAC vectors. The H2 was obtained by PCR from H2N3 AIV and cloned into pEAQ-HT. The H9 and H5 SUMO-tags were cloned into pEAQ-HT. All constructs were validated using Sanger sequencing and ready for transformation into *Agrobacterium tumefaciens* for plant infiltration.

3.1. Introduction

Plant molecular farming has recently become a significant application in applied biotechnology (Singh et al., 2021). Plant-based expression systems can be used for either transient or stable expression of recombinant proteins. Since it was established, a transient system is usually used due to reduced time and production costs (Sainsbury and Lomonossoff, 2008). The transient plant-based production system is regarded as the best expression system to produce vaccines by the Defense Advanced Research Projects Agency (DARPA) (Morris et al., 2016).

Stable transformation can be accomplished via nuclear or plastid integration (Altpeter et al., 2005). The desired gene is permanently inserted into the plant cell genome (Ma and Chen, 2005). Gene silencing induced by the plants as a defence affects the protein yield using a stable expression system. The disadvantage of this system is that it needs a long time, has low protein yield and random entry to the plant cell affects the transgene expression (Meriç et al., 2021).

In contrast, transient transformation allows protein production without integration into the plant cell genome. The transient expression system includes using *Agrobacterium*-mediated vector delivery containing the desired gene into plant cells for recombinant protein expression (Sainsbury and Lomonossoff, 2008). *Agrobacterium tumefaciens*, a soil bacterium responsible for plant tumour formation, has been altered to deliver the

desired gene without tumour formation (Hellens et al., 2000). The selection of the *Agrobacterium* strain is a significant factor affecting the target protein expression. Different selectable marker genes have been established in various disarmed *A. tumefaciens* strains suitable for transient plant-based expression (Hellens et al., 2000). Different *A. tumefaciens* strains have varying efficiencies depending on the target protein (Wroblewski et al., 2005, Wydro et al., 2006, Norkunas, 2014, Shamloul et al., 2014). Acetosyringone, a potent inducer of *Agrobacterium* virulence genes, can be added to the infiltration buffer to increase the transformation efficiency, increasing the expression of the recombinant protein (Norkunas, 2014, Pêra et al., 2015). The density of the *A. tumefaciens* culture is a crucial factor to consider. Low culture densities lead to low protein yield, while high densities may lead to necrosis of the leaves because of a hypersensitivity reaction (Wroblewski et al., 2005, Loh, 2014).

Agroinfiltration involves the introduction of *A. tumefaciens* containing an expression vector into the plant leaf by a syringe or using a vacuum. Agroinfiltration is a simple method that produces high protein yields in as little as 3 to 7 days (Pawar and Waghmare, 2016).

One of the biggest success stories of transient plant-based expression is the development of influenza vaccines. Vaccination against zoonotic diseases like influenza viruses supports One Health, a worldwide approach incorporating medicine and veterinary medicine to improve the health and welfare of all species (Mackenzie and Jeggo, 2019). In the case of viruses with a high mutation rate that necessitates frequent vaccine updates or, in

the event of a pandemic, the short production time of transient plant-based expression vaccines is a significant advantage allowing the rapid introduction of disease control by vaccination (D'Aoust et al., 2010).

3.2. Material and methods

3.2.1. Transformation of the verified constructed expression vectors into *Agrobacterium tumefaciens*

Two commonly used *A. tumefaciens* strains, GV3101 and LBA4404 (Hellens et al., 2000), were compared in this study. GV3101 was kindly provided by Dr Kostya Kanyuka (Rothamsted Research, UK), and ElectroMAX™ LBA4404 was purchased (Thermo Fisher Scientific™). After selecting positive recombinant clones by restriction digestion of the plasmid, electroporation (2500 V, 25 µF, 200 Ω) was applied to transform the target plasmid (100ng/ µl) into competent *A. tumefaciens*. The bacterial cells were re-suspended in 800 µl of LB Lennox and incubated for 1 hour at 28°C 225 rpm. Then, the transformed cells were spread-plated onto LB agar supplemented with (15 µg/ml rifampicin and 50 µg/ml kanamycin) and incubated for 3 days at 28◦C. Clones resistant to antibiotics were selected for overnight culture and plant infiltration.

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

Agroinfiltration was used to infiltrate *N. benthamiana* leaves with transformed *A. tumefaciens* strains GV3101 and LBA4404, containing the constructed plasmids (Shamloul et al., 2014). The transformed *A. tumefaciens* cultures were streaked onto LB agar containing 15 µg/ml

rifampicin and 50 µg/ml kanamycin. The LB broth containing 15 µg/ml rifampicin and 50 µg/ml kanamycin was inoculated with *Agrobacterium* colonies and incubated for 20 hours at 28°C 225 rpm on a shaking incubator. Overnight grown *Agrobacteria* were centrifuged (4,000 x g, 5 minutes), and the pellets suspended in infiltration buffer (10 mM 2-N-morpholino-ethane sulfonic acid (MES), 10 mM $MgCl_2$, pH 5.6) supplemented with 0.1 mM acetosyringone. The individual infiltration mixes were diluted to obtain a final $OD_{600\ nm}$ 0.5. Following incubation at room temperature for three hours on the bench, the *A. tumefaciens* suspensions were introduced into *N. benthamiana* leaves (Strasser et al., 2008) using a syringe.

3.2.3. Harvesting of infiltrated leaf material and total protein extraction

At seven days post infiltration (dpi), homogenization was used to extract the total soluble protein from the plant tissue, and the target protein was purified. Two volumes of cold protein extraction buffer (50 mM Tris, 500 mM NaCl, 10 mM sodium thiosulphate, and 0.1% Triton X-100, pH 7.2) according to Prof. George Lomonossoff protocol. Before use, the extraction buffer was supplemented with a Protease Inhibitor Cocktail (EDTA-Free, 100X in DMSO) (B14002, bimake.com) to prevent proteolysis of the target protein. After homogenization, the tissue slurries were filtered through a double layer of Mira cloth, and the plant extract was centrifuged (10,000 x g, 10 minutes, 4°C). Target proteins were subsequently purified using immobilised metal affinity chromatography (IMAC). The ProBond™ Purification System (ThermoFisher Scientific, UK) is designed to purify His-tagged recombinant

proteins using nickel-chelating resin. First, the ProBond™ resin was used according to the manufacturer's protocol. For the VLP extraction, the VLP extraction buffer was modified (50 mM Tris, 75 mM NaCl, and 0.1% Triton X-100). Target VLPs were subsequently purified using centrifugation. The clear supernatant containing H9 or H5 VLPs was loaded on top of a freshly prepared 30 and 70% sucrose density cushion. Layers were 2 ml each, and ultracentrifugation was performed (32,000 x g, 3 hours, 4°C; Hitachi Ultra-centrifuge CP 80NX), fractions of 70% layer, interface, 30% layer, and the supernatant were collected.

3.2.4. Biochemical analysis and confirmation of identity

3.2.4.1. Immobilised metal affinity chromatography

The ProBond™ Purification System (Thermo Fisher Scientific, UK) is designed to purify His-tagged recombinant proteins using nickel-chelating resin. First, the ProBond™ resin was resuspended in its bottle by inverting repeatedly. The resin (3 ml) was poured into a 10-ml Purification Column supplied with the kit. The resin was allowed to settle entirely by gravity (5–10 minutes) or was gently pelleted by low-speed centrifugation (1 minute at 800 × g). The supernatant was gently aspirated, 6 ml of sterile distilled water was added, and the resin was resuspended by inverting and gently tapping the column.

The resin was settled using gravity or centrifugation, and the supernatant was gently aspirated. For the purification under native conditions, 6 ml of the ProBond™ Native Binding Buffer was added, and the resin was resuspended

by alternately inverting and gently tapping the column. The resin was settled using gravity or centrifugation as described before, the supernatant was gently aspirated, and the washing step was repeated. The lysate prepared under native conditions (7 ml) was added to a prepared Purification Column and left to bind for 30–60 minutes using gentle agitation to keep the resin suspended in the lysate solution. The resin was settled by gravity or low-speed centrifugation (800 × g), and the supernatant was allowed to flow through the column. The resin was washed with 8 ml ProBond™ Native Wash Buffer and was settled through gravity or low-speed centrifugation (800 × g), and the supernatant was allowed to flow through the column. The washing step was repeated three more times. The column was clamped vertically and snapped off the lower-end cap. The protein was eluted with 8–12 ml of ProBond™ Native Elution Buffer.

3.2.4.2. Bradford assay

Bradford assay was done to detect the amount of proteins. Pierce™ Coomassie Plus (Bradford) Assay Kit (catalogue number 23236, Thermo Fisher Scientific, UK) was used following the manufacturer's guidelines. Bovine serum albumin (BSA) was used as standard at concentrations in mg/ml (2, 1.5, 0.5, 0.25, 0.125) to measure the concentration of proteins in the sample.

3.2.4.3. Gel electrophoresis and immunoblotting

The eluted protein was analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis. For SDS-PAGE, the protein was separated on a 12% Mini-PROTEAN® TGX™ Precast Protein Gels (4561043, Bio-Rad, UK) under reducing conditions by using 4x reducing Laemmli buffer and stained with SimplyBlue™ SafeStain (LC6060, Thermo Fisher Scientific, UK). The prestained ladders used are Amersham ECL High-Range Rainbow Molecular Weight Markers (reference RPN756E, Cytvia) and Precision Plus Protein Dual Colour Standards (reference 1610374, Bio-Rad).

For western blotting analysis, eluted His tagged proteins or VLPs were separated on a 12% Mini-PROTEAN® TGX™ Precast Protein Gels under reducing conditions by using 4x reducing Laemmli buffer and colour pre-stained protein standard (P7719, New England Biolabs, UK) and transferred to Trans-Blot Turbo Mini 0.2 µm Nitrocellulose Transfer Packs (Cataloug no. 1704158, Bio-Rad, UK) using the semi-dry transblot Turbo blotter (Bio-Rad), according to the manufacturer's recommendation. Blocking was performed in 5% skimmed milk (used with H9 and H5 antibodies) or 5% bovine serum albumin (BSA) (used with 6 His-tag antibodies) for one hour. H9 purified protein was detected using rabbit anti-influenza A virus H9N2 HA antibody (1:2000, GTX127305-GTX, Stratech, UK) and anti-rabbit IgG horseradish peroxidase (HRP) linked antibody (1:5000, catalouge no. 7074S, Cell Signaling Technology, UK). H5 purified protein was detected using influenza A virus H5N8 HA antibody (1:2000, 11717-RP01-SIB, Stratech, UK) and

anti-rabbit IgG horseradish peroxidase (HRP) linked antibody (1:5000, catalouge no. 7074S, Cell Signaling Technology, UK). 6x-His-tag monoclonal (HRP) conjugated antibody (1:2000, MA1-21315-HRP, ThermoFisher, UK) or tetra His-tag antibody (1:1000, catalogue number 34670. Qiagen, UK) was used to detect the purified H5 and H9 proteins. Anti-mouse HRP-linked IgG Antibody (catalogue number 7076S, Cell Signaling Technology, Netherlands) was used as a secondary antibody with the tetra His-tag antibody. Chemiluminescence detection (Amersham™ ECL Prime Western Blotting Detection Reagent, 12316992, GE Healthcare) and the ChemiDoc™ MP Imaging System (Bio-Rad) was used for protein visualization, as recommended by the manufacturer.

3.2.4.4. Transmission electron microscopy (TEM)

To confirm the formation of HA-based VLPs, partially-purified H9 and H5 VLPs were prepared for examination by negative-staining transmission electron microscopy (TEM) (A JEOL 2100+TEM, Electron Microscopy Unit, nmRC, University of Nottingham). Concentrated VLPs (20 µl) were incubated on carbon-coated copper grids (mesh size 200) for 10 minutes and washed with 5 µl sterile water for three washes. Particles were subsequently negatively stained with 2% uranyl acetate for 60 seconds before imaging.

3.2.4.5. Haemagglutination assay

Haemagglutination assay (HA) for H9 and H5 VLPs was performed based on the method explained by Nayak and Reichl (2004). Two-fold serial dilutions of the VLPs (50 µl) were prepared in 96 well V− bottom microtitre plates, diluted in 50 µl phosphate-buffered saline (PBS), finishing with 50 µl of diluted sample per well. Washed 1% chicken red blood cell suspension (50 µl) (TCS Biosciences Ltd, UK) was added to all wells, and the plates were left for two hours at room temperature. The titre was recorded, which is the reciprocal of the highest dilution of the H9 revealing complete haemagglutination. A negative control plant lysate infiltrated with an empty pEAQ-HT vector was diluted in used as a negative control on each plate.

3.3. Results

3.3.1. Transient gene expression in the *Nicotiana benthamiana*

A. tumefaciens GV3101 overnight culture containing pEAQ-HT-H9-VLP gave an OD_{600} of 1.0−1.5, while *A. tumefaciens* LBA14404 containing the same construct gave an OD_{600} of 0.1−0.2.

A. tumefaciens cultures containing pEAQ-HT HA construct and pEAQ-HT GFP were syringe infiltrated into *N. benthamiana* leaves. Leaf tissues were harvested eight days post-infiltration and stored at -80°C (Figure 3.1). Leaves containing pEAQ-HT GFP were analysed under a U.V transilluminator, and green fluorescence colour due to the presence of GFP was demonstrated (Figure 3.2).



Figure 3.1. Harvested *N. benthamiana* leaves infiltrated with *A. tumefaciens* bacteria containing H9N2 HA construct. The infiltrated leaves on the right side were compared to the negative control leaves on the left side and both harvested 7 dpi. The infiltrated leaves did not show any necrosis.

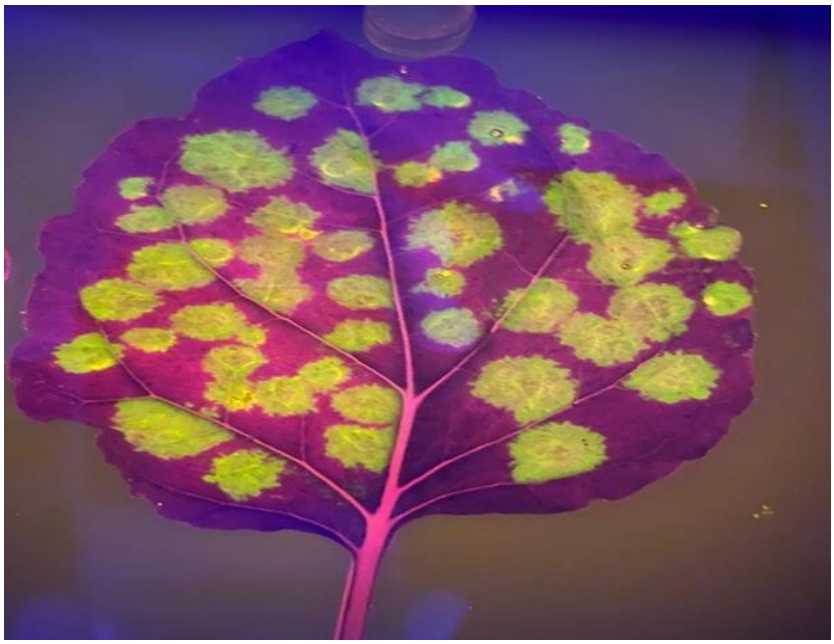


Figure 3.2. Examination of the infiltrated *N. benthamiana* plant with *A. tumefaciens* containing pEAQ-HT vector with GFP under UV transilluminator

appears fluorescent green. The pEAQ-HT vector with GFP was used as a positive control and showed successful infiltration.

3.3.2. Expression of recombinant proteins and VLPs

3.3.2.1. Bradford assay

Bradford was used to detect the amount of proteins expressed in plants. The plant lysate containing H9 VLPs gave 2.1 mg/ml, while the H5 VLPs gave 1.9 mg/ml compared to the negative control plant lysate, which contained 1.3 mg/ml. The results showed that the H9 and H5 VLPs were expressed in plants.

3.3.2.2. Expression of pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag proteins

The H9 and H5 plant lysate and purified proteins were analysed using stained SDS-PAGE. Stained protein gel for the pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag proteins showed bands at around 65.5 and 66.6 kDa, respectively (Figure 3.3).

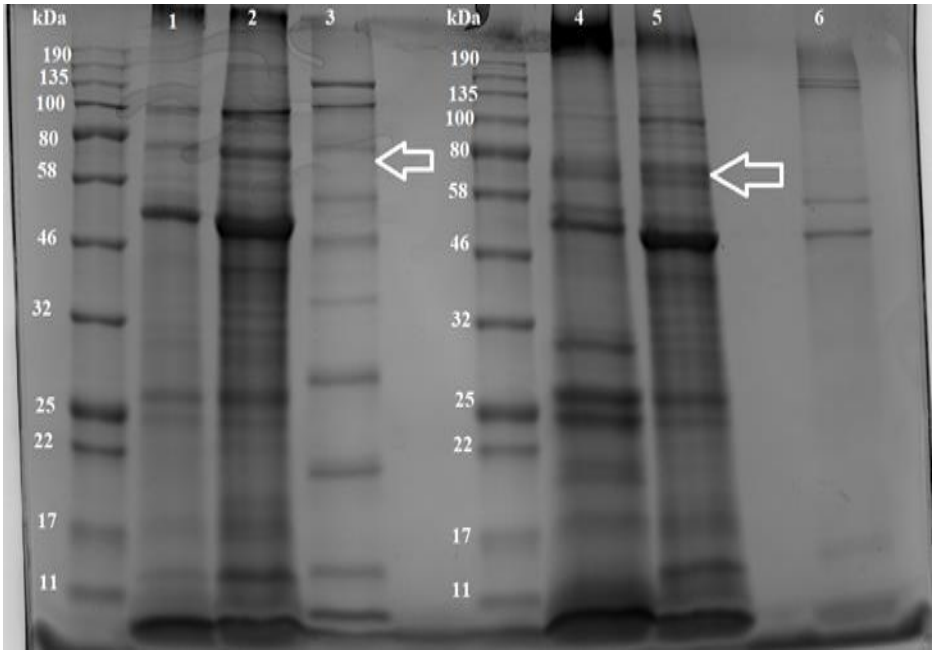


Figure 3.3. Stained SDS PAGE for the expression of pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag proteins. Lane 1 represents the H9 plant pellet. Lane 2 is the H9 plant lysate, Lane 3 is the H9 plant lysate flow-through, and all showed bands at the expected size of around 65.6 kDa, as represented with the arrow. For the H5 plant proteins, Lane 4 represents the H5 plant pellet, Lane 2 is the H5 plant lysate, and all showed bands at the expected size around 66.6 kDa as represented with the arrow, and Lane 6 is the negative control plant lysate.

Purification of pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag proteins using IMAC. The eluted H9 and H5 proteins were partially purified and a low amount of purified proteins were detected (Figure 3.4).

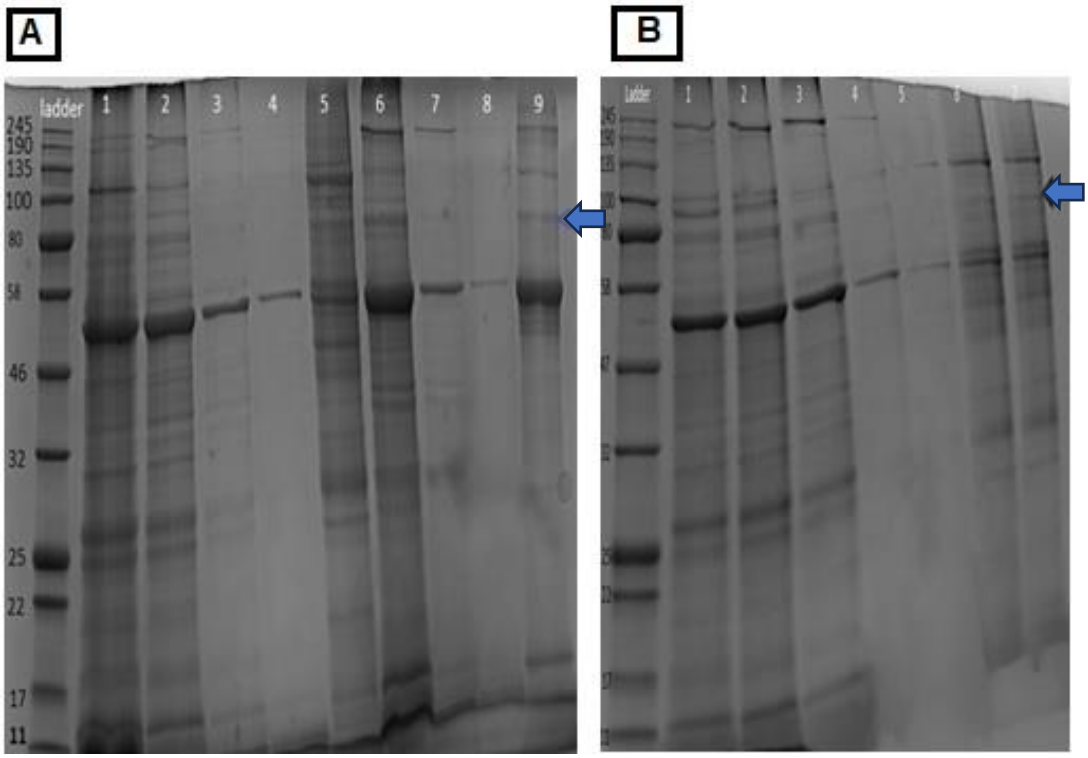


Figure 3.4. Stained SDS PAGE for the pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag protein purification. Image A showed the purification of pEAQ-HT-H9-His-tag proteins. Lane 1 represents the H9 plant lysate, lane 2 is the H9 flow-through, Lane 3 is the first wash, lane 4 is the second wash, lane 5 is the lysate after syringe filtration, lane 6 is the purified H9 protein, lane 7 is the third wash, lane 8 is the fourth wash, and lane 9 is the elution. All showed the band at the expected size of around 65.6 kDa, as represented by the arrow. Image B showed the pEAQ-HT-H5-His-tag protein purification. Lane 1 represents the H5 plant lysate, lane 2 is the purified H5 lysate, lane 3 is the H5 flow-through, lane 4 is the first wash, lane 5 is the second wash, lane 6 is the first elution, and lane 7 is the second elution. All showed the band at the expected size of around 66.6 kDa, as represented by the arrow.

Western blot for the pEAQ-HT-H9-His-tag proteins, using an anti-H9 antibody and anti-His-tag antibody, showed bands at 65.6 (Figure 3.5). Western blot showed a good expression level in the plant lysate and a lower quantity of proteins in the fractions purified using IMAC. The western blot showed HA monomers, dimers, and trimers expression pattern bands. A negative control sample containing plant lysate infiltrated with an empty pEAQ-HT vector was validated and confirmed no reaction with the antibody.

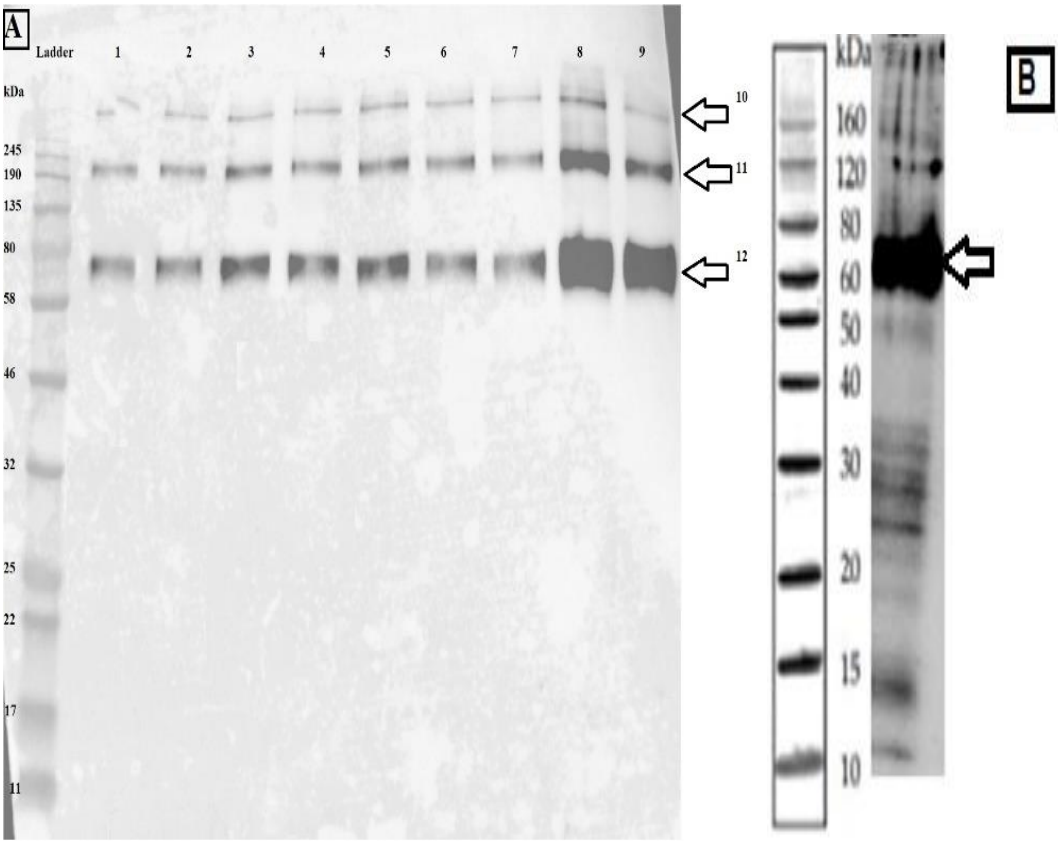


Figure 3.5. Western blot for the pEAQ-HT-H9-His-tag proteins. Image A shows the western blotting for the pEAQ-HT-H9-His-tag proteins using anti H9 antibody. Lanes (1–7) represent the purified H9 protein elutions, lane 8 is the H9 plant lysate, and lane 9 is the H9 plant pellet of around 65.6 kDa, as represented with the arrow. Bands (10–12) represent the HA monomers,

dimers, and trimers, respectively. Image B showed the western blotting for pEAQ-HT-H9-His-tag plant lysate protein using an anti-His-tag antibody and showed a band at the expected size of around 65.6 kDa, as represented by the arrow. A negative control sample containing plant lysate infiltrated with an empty pEAQ-HT vector was validated and confirmed no reaction with the antibody.

Western blot for the pEAQ-HT-H5-His-tag proteins, using an anti-H5 antibody and anti-His-tag antibody, showed bands at 66.6 kDa (Figure 3.6). The western blot showed HA monomers, dimers, and trimers expression pattern bands. Usually, some proteins were degraded, as seen in Figure A; some proteins at 58 kDa represented some degradation to the H5 proteins (Figure A).

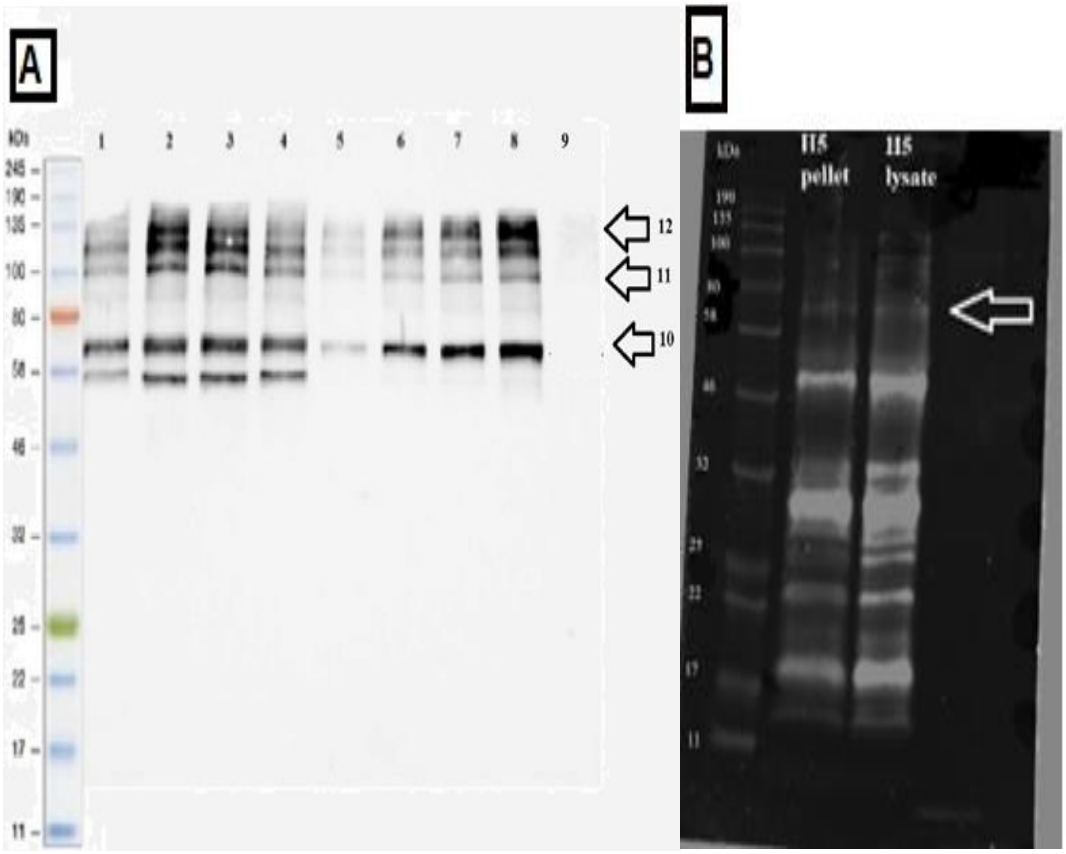


Figure 3.6. Western blot for the pEAQ-HT-H5-His-tag proteins. Image A showed the pEAQ-HT-H5-His-tag proteins detected using anti H5 antibody. Lane 1 represents the H5 plant lysate; lane 2 is the H5 plant pellet; Lane 3 is H5 lysate after syringe filtration; lane 4 is H5 lysate after binding to the resin, lane (5–8) is the purified H5 protein, and lane 9 represents the negative control lysate. All showed the band at the expected size of around 66.6 kDa, as represented by the arrow. Bands (10–12) represent the HA monomers, dimers, and trimers, respectively. Image B showed the pEAQ-HT-H5-His-tag plant lysate protein using an anti-His-tag antibody and showed bands at the expected size of around 66.6 kDa, as represented by the arrow.

3.3.2.3. Expression of pEAQ-HT-H9-VLPs

The pEAQ-HT-H9-VLPs were purified via ultracentrifugation and showed bands at around 65.5 kDa (Figure 3.7). H9 VLPs showed dimers and trimers expression patterns.

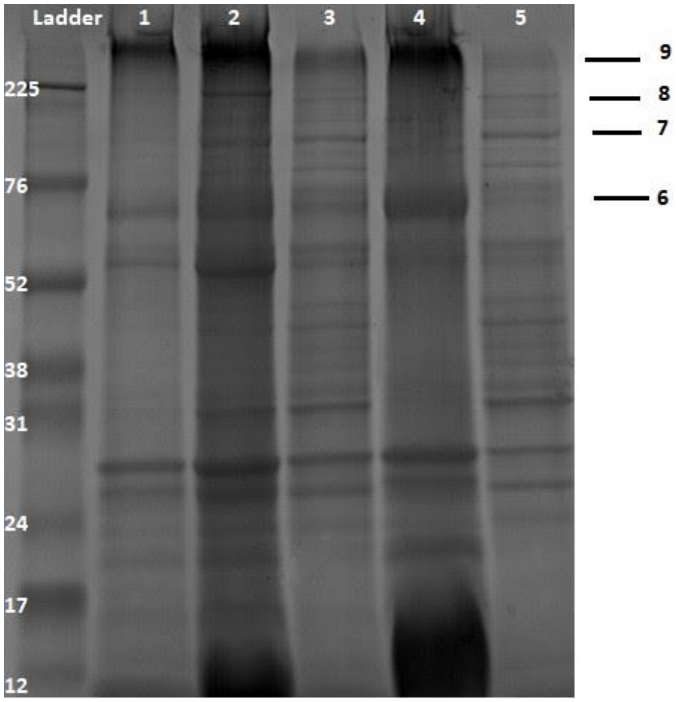


Figure 3.7. Stained SDS PAGE of pEAQ-HT-H9-VLPs purified via sucrose cushion ultracentrifugation. Lane 1 represents the pellet left after protein extraction, while lane 2 represents the plant lysate. Lane 3 represents the syringe-filtered plant lysate. Lane 4 represents the concentrated VLPs by ultracentrifugation, while lane 5 represents the collected supernatant after ultracentrifugation. Bands (6–9) represent the H9 monomer, dimer, trimer, and VLP bands.

pEAQ-HT-H9-VLPs expression was confirmed using a specific anti-H9 antibody. VLPs were extracted from the plants, purified, and concentrated using sucrose gradient ultracentrifugation (Figure 3.8). A negative control sample containing plant lysate infiltrated with an empty pEAQ-HT vector was validated and confirmed no reaction with the antibody.

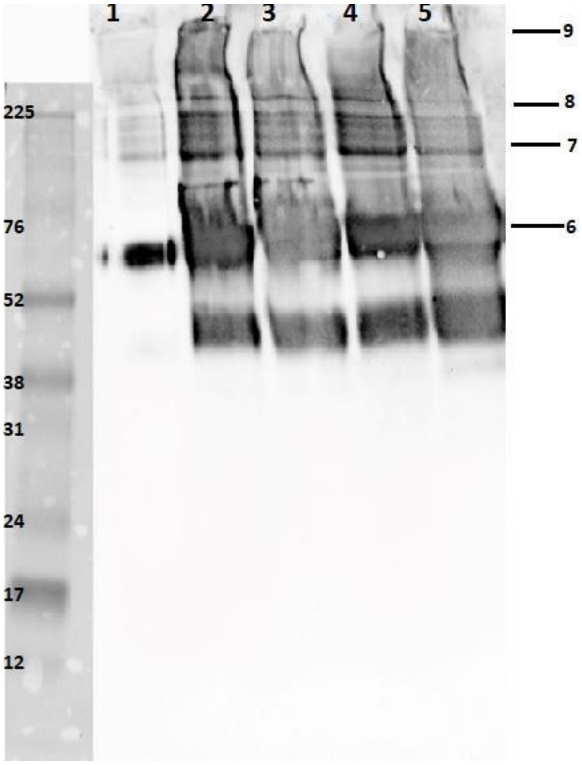


Figure 3.8. Western blot of pEAQ-HT-H9-VLPs purified via sucrose cushion ultracentrifugation. H9 VLP expression was confirmed using a specific anti-H9 antibody. Lane 1 represents the pellet left after protein extraction, while lane 2 represents the plant lysate. Lane 3 represents the syringe-filtered plant lysate. Lane 4 represents the concentrated VLPs by ultracentrifugation, while lane 5 represents the collected supernatant after ultracentrifugation. Bands 6-9 represent the H9 monomer, dimer, trimer, and VLP bands, as represented by lines.

3.3.2.4. Expression of pEAQ-HT-H5-VLP

Stained gel electrophoresis for the pEAQ-HT-H5-VLP purification via ultracentrifugation showed bands at around 66.6 kDa (Figure 3.9). H5 VLPs showed dimers and trimers expression patterns.

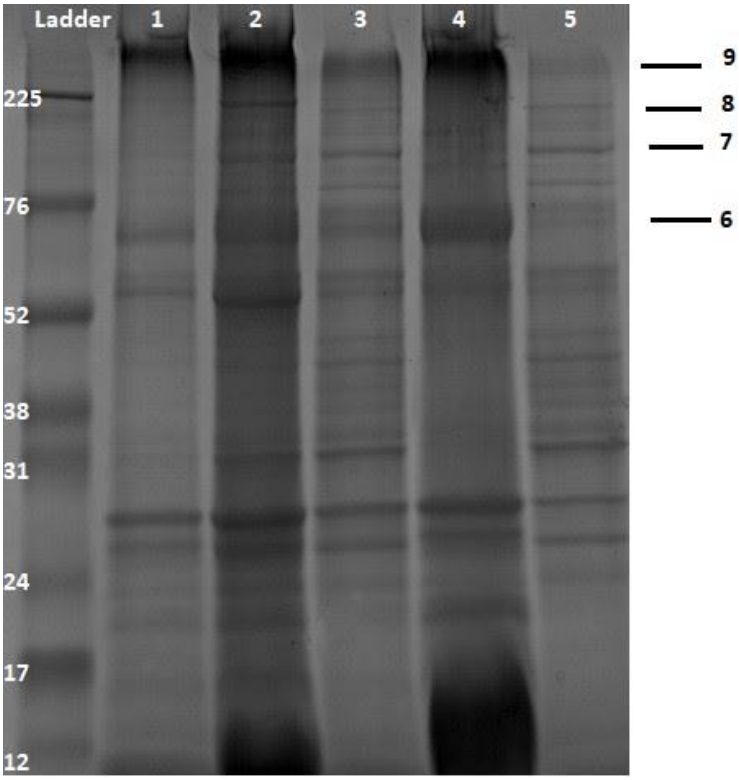


Figure 3.9. Stained SDS PAGE of pEAQ-HT-H5-VLP purification via sucrose cushion ultracentrifugation. Lane 1 represents the pellet left after protein extraction, while lane 2 represents the plant lysate. Lane 3 represents the syringe-filtered plant lysate. Lane 4 represents the concentrated VLPs by ultracentrifugation, while lane 5 represents the collected supernatant after ultracentrifugation. Bands (6–9) represent the H5 monomer, dimer, trimer, and VLP bands, as represented by lines.

pEAQ-HT-H5-VLP expression was confirmed using a specific anti-H5 antibody. VLPs were extracted from the plants, purified, and concentrated using sucrose cushion ultracentrifugation (Figure 3.10). A negative control sample containing plant lysate infiltrated with an empty pEAQ-HT vector was validated and confirmed no reaction with the antibody.

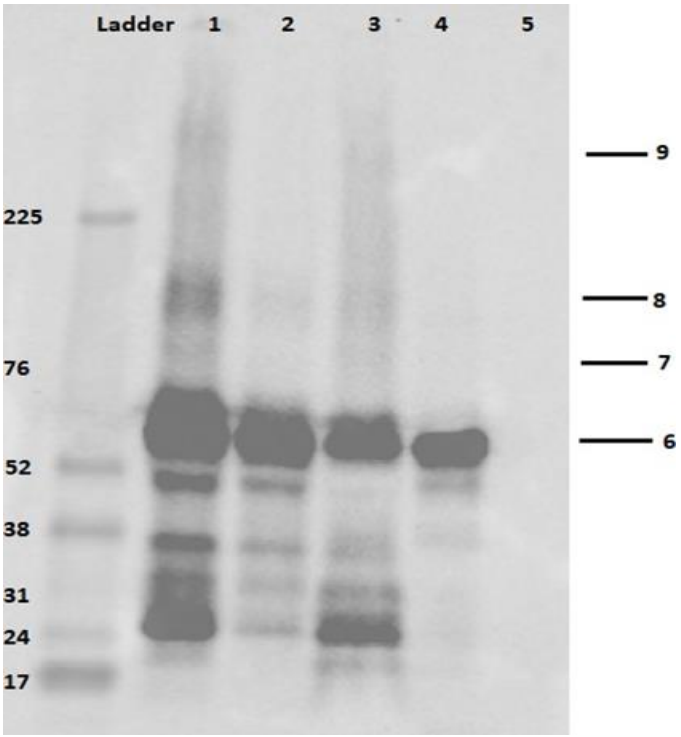


Figure 3.10. Western blot of pEAQ-HT-H5-VLP purification via sucrose cushion ultracentrifugation. H5 VLP expression was confirmed using a specific anti-H5 antibody. Lane 1 represents the plant lysate. Lane 2 represents the syringe-filtered plant lysate. Lane 3 represents the concentrated VLPs by ultracentrifugation, while lane 4 represents the collected supernatant after ultracentrifugation. Lane 5 represents the negative control plant lysate. Bands 6-9 represent the H5 monomer, dimer, trimer, and VLP bands, as represented by lines.

3.3.2.5. Expression of pHREAC-H9-His-tag protein

pHREAC-H9-His-tag proteins were expressed in plants using the pHREAC vector and showed similar expression levels for H9 proteins using the pEAQ-HT vector, depending on the band intensity (Figure 3.11).

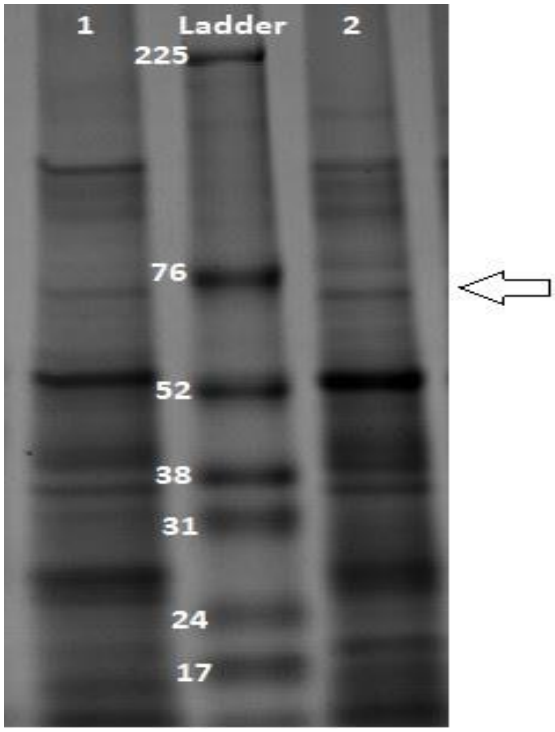


Figure 3.11. Stained SDS PAGE to compare the expression level of H9-His-tag protein produced using pEAQ-HT and pHREAC vectors. Lane 1 represents pHREAC-H9 His-tagged proteins, while lane 2 represents pEAQ-HT-H9 His-tagged proteins; both were detected at the expected size of 65.6 kDa, as represented by the arrow. A similar expression level was detected using the two vectors.

The pHREAC-H9- His-tag protein was also successfully expressed using the pHREAC vector (Figure 3.12). The western blot showed HA monomers, dimers, and trimers expression pattern bands.

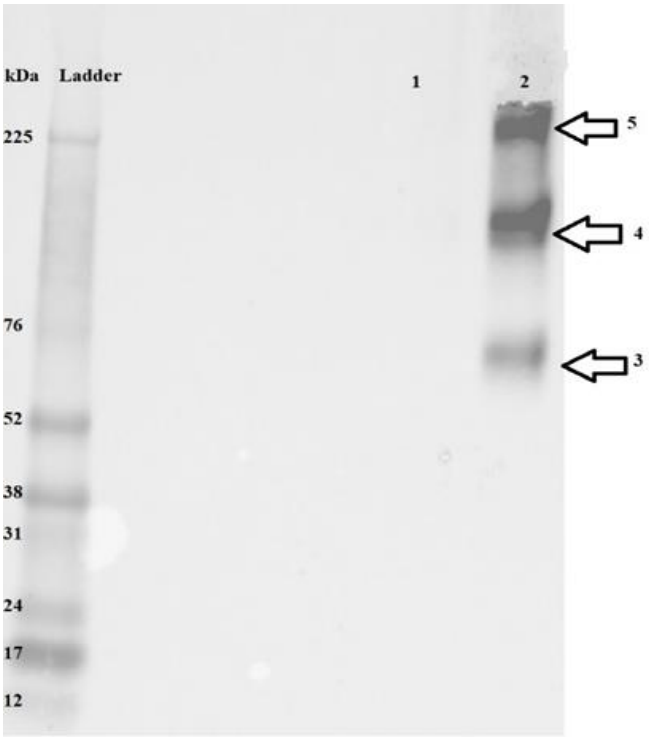


Figure 3.12. Western blot of pHREAC-H9- His-tag protein confirmed using a specific anti-H9 antibody. Lane 1 represents the negative control lysate, and lane 2 represents the H9 plant lysate which showed a band of around 65.6 kDa. Bands (3–5) represent the HA monomers, dimers, and trimers, as represented by arrows.

3.3.2.6. Expression of pEAQ-HT-H2-His-tag protein

The H2 plant lysate and purified proteins were analysed on the gel. The pEAQ-HT-H2-His-tag was expressed in plants and showed bands at around 63.8 kDa (Figure 3.13).

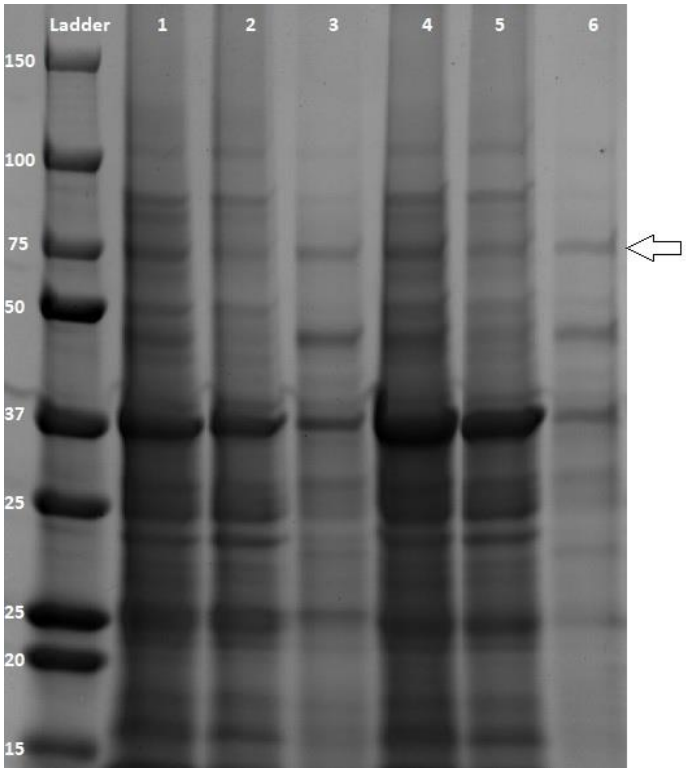


Figure 3.13. Stained SDS PAGE of pEAQ-HT-H2-His-tag protein. Lanes (1,4) represent the pellet from different batches after protein extraction, and lanes (2,5) represent the plant lysate. Lanes (3,6) represent the purified protein elution. All showed bands at the expected size of around 63.8 kDa, as represented by the arrow.

pEAQ-HT-H2-His-tag proteins were expressed and confirmed using an anti-His-tag antibody at the expected size of around 64 kDa (Figure 3.14). The amount of protein detected in the plant lysate was low, but a good amount

of purified proteins were found in the elution. A negative control sample containing plant lysate infiltrated with an empty pEAQ-HT vector was validated and confirmed no reaction with the antibody.

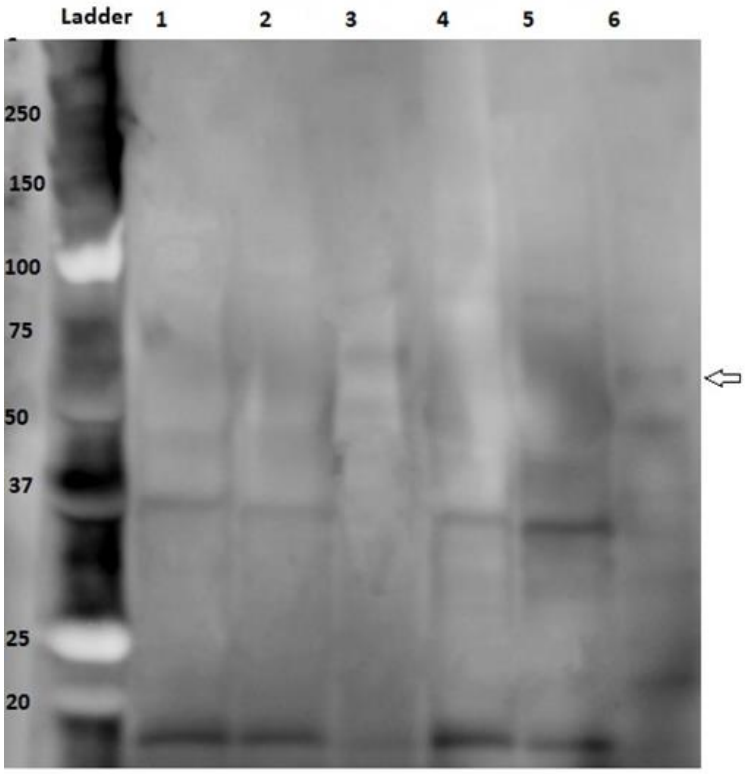


Figure 3.14. Western blot of pEAQ-HT-H2-His-tag protein confirmed using tetra His-tag antibody. Lanes (1,4) represent the pellet from different batches after protein extraction, while Lanes (2,5) represent the plant lysate. Lanes (3,6) represent the purified protein elution. All showed a band at the expected size of around 64 kDa, as represented by the arrow.

3.3.2.7. Expression of pEAQ-HT-H9-SUMO-tag and pEAQ-HT-H5-SUMO-tag proteins

The pEAQ-HT-H9-SUMO-tag and pEAQ-HT-H5-SUMO-tag proteins were purified using IMAC. After cloning into pEAQ-HT using *AgeI* and *XmaI,* leaving an *XmaI* restriction site between the construct and the His-tag, a high amount of protein was detected in the purified elutions depending on the band intensity. Monomers are only detected after deleting the TMD (Figure 3.15).

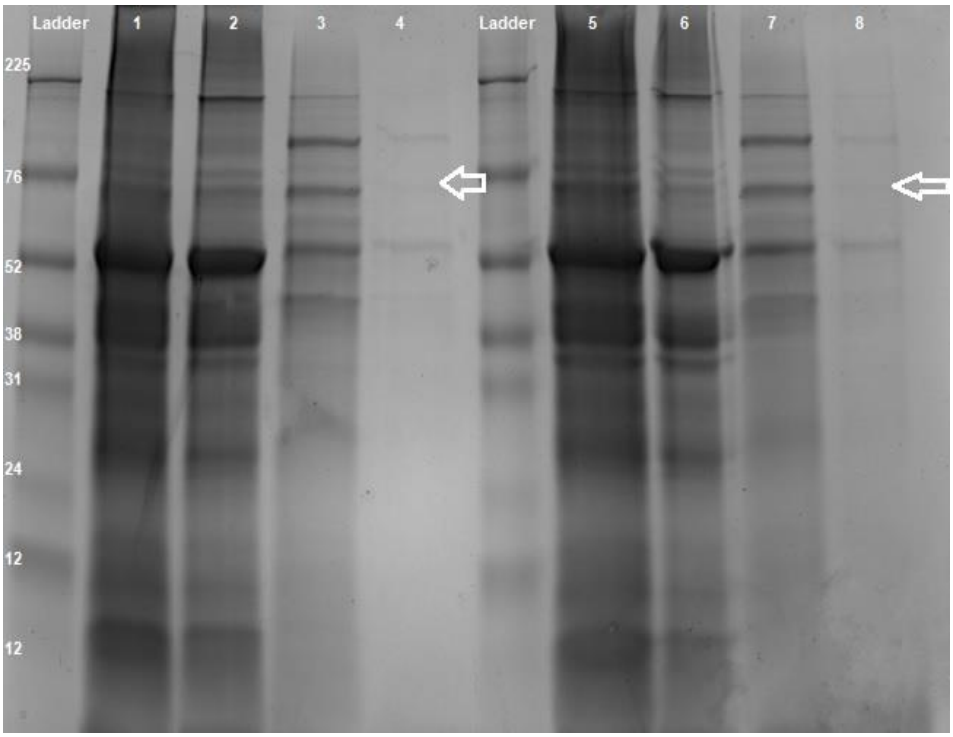


Figure 3.15. Stained SDS PAGE for the pEAQ-HT-H9-SUMO-tag and pEAQ-HT-H5-SUMO-tag protein purification. Lane 1 represents the H9 plant lysate, Lane 2 is the H9 flow-through, Lane 3 is the first elution, and Lane 4 is the second elution. All showed the band at the expected size of around 72.6 kDa, as represented by the arrow. Lane 5 is the H5 plant lysate, lane 6

is the H5 flow-through, lane 7 is the first elution, and lane 8 is the second elution. All showed the band at the expected size of around 73.7 kDa, as represented by the arrow.

pEAQ-HT-H9-SUMO-tag and pEAQ-HT-H5-SUMO-tag proteins were detected using tetra His-tag antibody. HA Monomers are only detected after deleting the TMD (Figure 3.16).

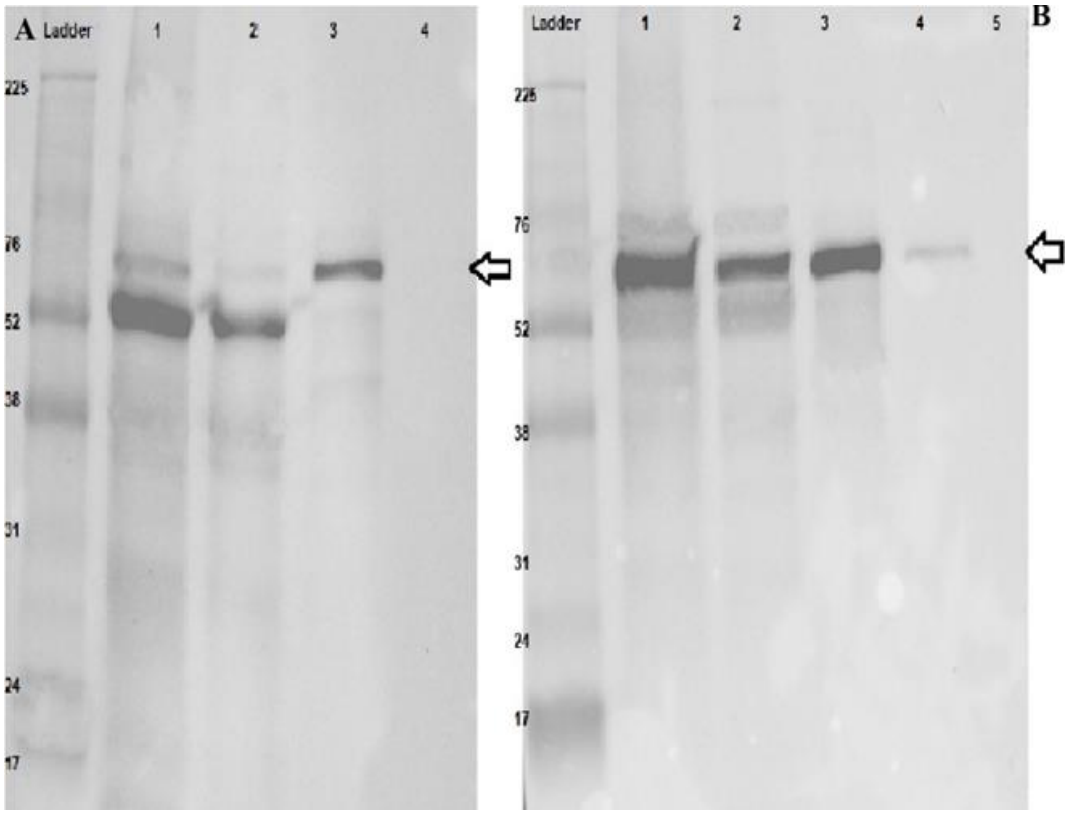
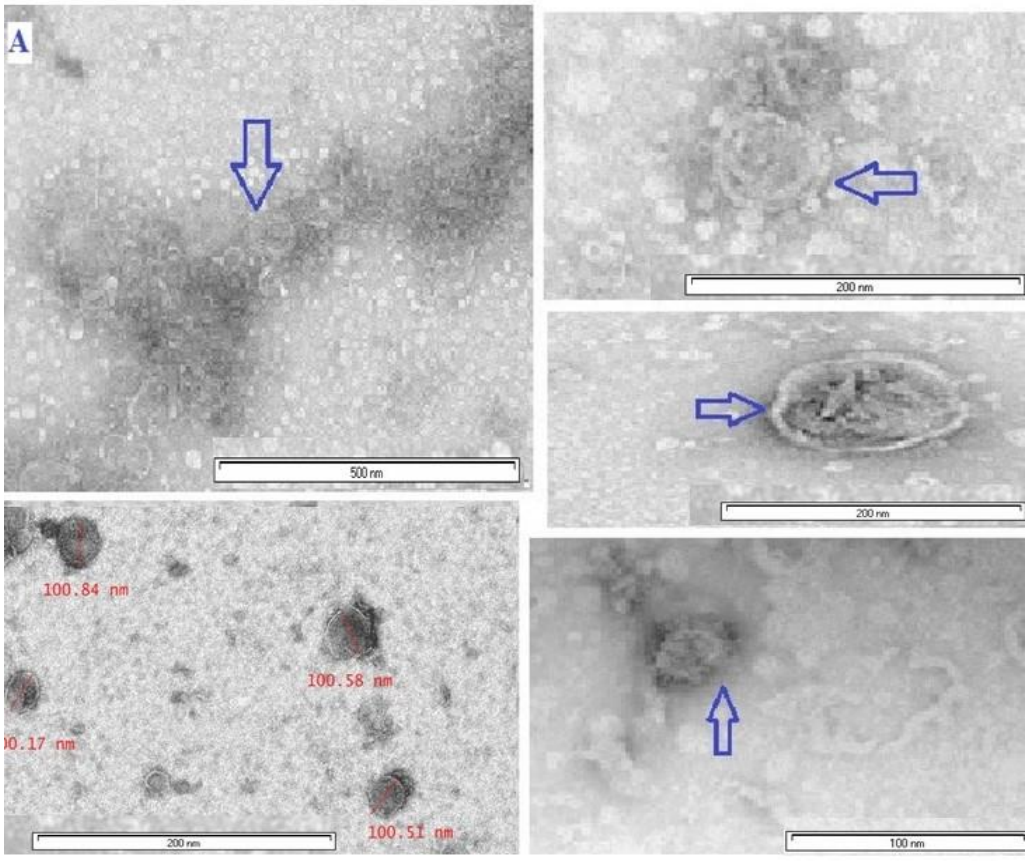


Figure 3.16. Western blot of the pEAQ-HT-H9-SUMO-tag and pEAQ-HT-H5-SUMO-tag proteins using tetra His-tag antibody. Lane 1 represents the H9 plant lysate, Lane 2 is the H9 flow-through, Lane 3 is the first elution, and Lane 4 is the negative control lysate. The H9-SUMO-tagged proteins showed the band at the expected size of around 72.6 kDa, as represented

by the arrow. Lane 5 is the H5 plant lysate, lane 6 is the H5 flow-through, lane 7 is the first elution, and lane 8 is the negative control lysate. The H5-SUMO-tagged proteins showed the band at the expected size of around 73.7 kDa, as represented by the arrow.

3.3.3. Transmission electron microscope

TEM confirmed the presence of the H9 and H5 VLP, measuring around 100 nm (Figure 3.17). The H9 and H5 VLPs were roughly spherical, and particle size was around 100 nm using TEM, similar to previous results regarding influenza VLPs produced in plants (D'Aoust et al., 2008, Lindsay et al., 2018).



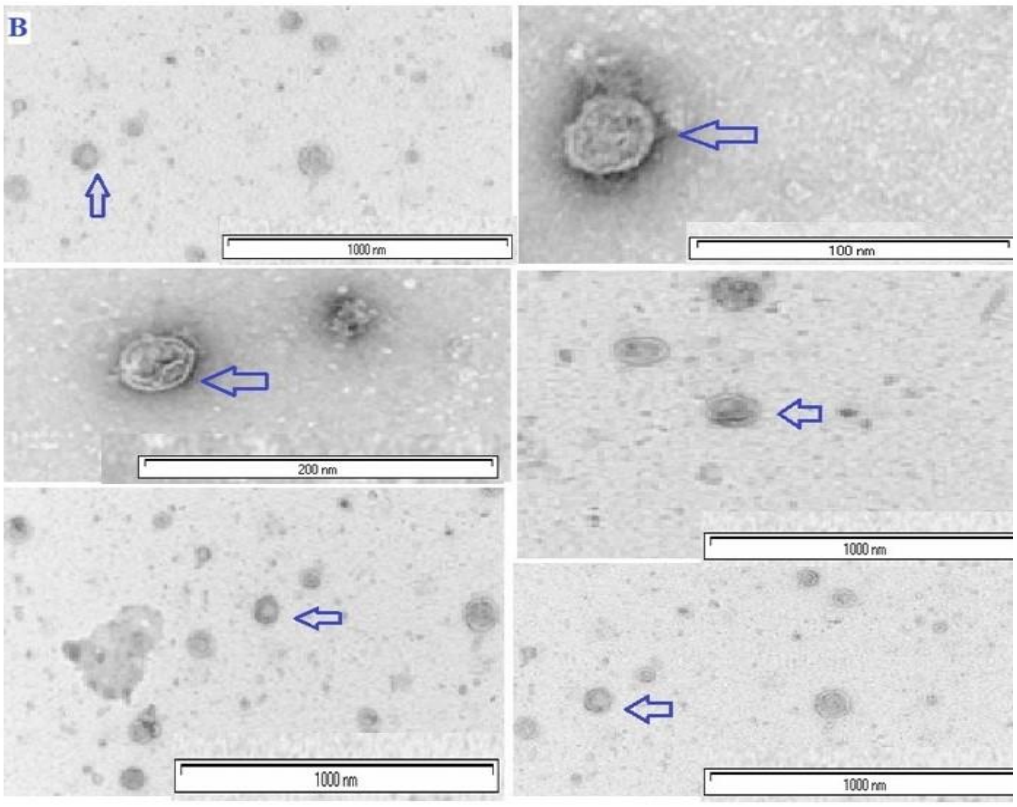


Figure 3.17. Negatively-stained transmission electron microscopy image of H9 and H5 VLPs. Image A: H9 VLPs and B: H5 VLPs. The arrows indicate the influenza VLPs.

3.3.4. Haemagglutination test

HA test was performed using concentrated pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP, and the results showed that the titre of the same pEAQ-HT-H5-VLP samples expressed in different plant batches showed agglutination of the chicken RBCs ranged from 128 to 256, while the titre of pEAQ-HT-H9-VLP samples using the VLP extraction buffer ranging from 128 to 512 (Figure 3.18). The control plant lysate containing no HA protein showed no agglutination of the RBCs.

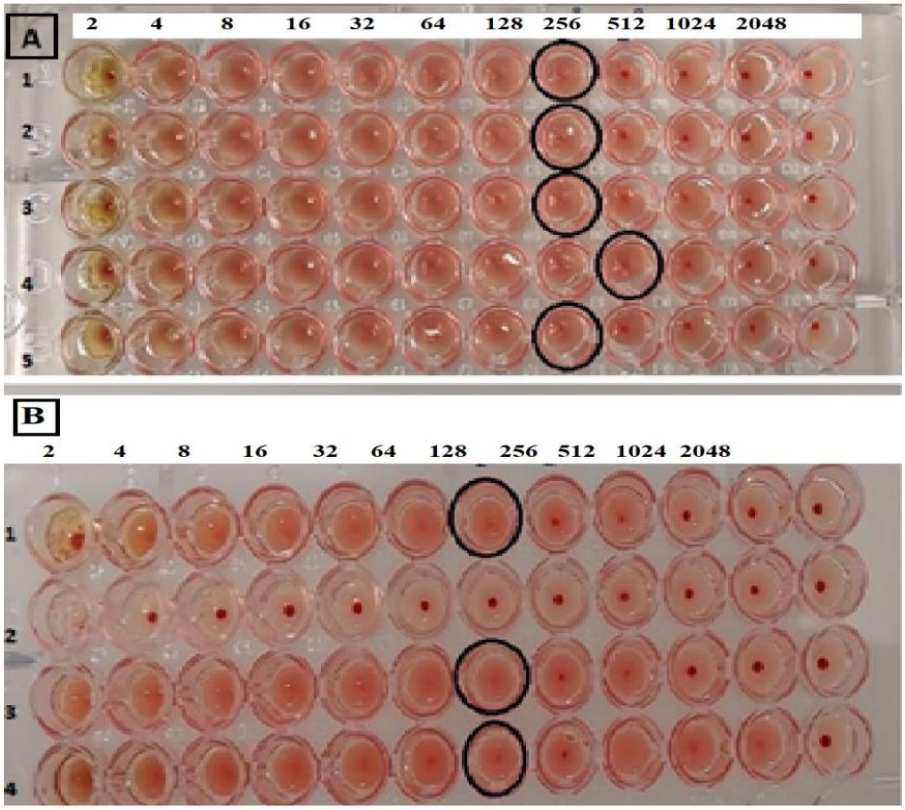


Figure 3.18. Haemagglutination test of pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP. In Figure A, lanes (1–3) represent concentrated pEAQ-HT-H9-VLP titre from different batches, while lanes (4,5) represent the same pEAQ-HT-H5-VLP expressed from different batches. In Figure B, lane 1 represents pEAQ-HT-H9-VLP, while lanes (3,4) represent pEAQ-HT-H5-VLP. Lane 2 represents negative control plant lysate. The HA titre was recorded on the top of the wells and the circle around the wells refers to the titre of each sample.

3.4. Discussion

pEAQ-HT-H9-His-tag, pEAQ-HT-H5-His-tag, and pEAQ-HT-H2-His-tag proteins were successfully expressed in *N.benthamiana*. pEAQ-HT-H2-His-tag recombinant protein was expressed in *N. benthamiana* to determine whether the codon optimisation is essential for protein expression and showed lower expression levels than pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag. H5 recombinant proteins against the H5N1 AIV virus have been successfully produced in *N. benthamiana* in many studies (Musiychuk et al., 2007, Spitsin et al., 2009, Mortimer et al., 2012, Major et al., 2015, Ceballo et al., 2017, Farsad et al., 2017, Pua et al., 2017, Phan et al., 2018, Thi Pham et al., 2019, Phan et al., 2020, Van et al., 2020). *Agrobacterium* GV3101 showed higher transformation efficiency recorded using OD_{600nm} and expression levels than *Agrobacterium* LBA14404, which agrees with previous reports (Liu et al., 2020).

Western blot of the pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP showed a pattern of expression as dimers, trimers, and VLPs; this could be because of two reasons. The first is that target proteins may be cross-linked and resistant to denaturation induced by some components in the plant extract, such as peroxidase, during the purification process (Castells-Graells and Lomonossoff, 2021). Secondly, when the same target proteins were expressed after the TMD deletion in the SUMO-tagged proteins, these patterns no longer existed, suggesting that TMD is responsible for the

trimerisation of the target proteins as shown in the H9 and H5 VLPs and His-tagged proteins.

The Kozak consensus sequence is commonly inserted before the start codon to improve translational efficiency (Kozak, 1987). Kozak sequence was added upstream of the start codon in all the recombinant proteins and VLPs. The codon-optimised pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag proteins were expressed more than the non-codon-optimised pEAQ-HT-H2-His-tag proteins. It is not expected that the Kozak sequence had a negative effect on the protein expression in this study.

The 6 x His-tag was added to the C-terminus of all our proteins to ease the purification without affecting the protein expression. Adding an N-His-tag could have either a detrimental or positive effect on protein expression (Svensson et al., 2006, Park et al., 2015, Manuel-Cabrera et al., 2016, Booth et al., 2018).

IMAC is one of the techniques frequently used to purify recombinant proteins (Kuo and Chase, 2011, Zhao et al., 2019). The side chains of some amino acids, particularly histidines, on the surface of the target protein interact with immobilised metal ions, particularly Ni^{2+}, to enable purification. The purified target molecule is then recovered from the matrix by elution buffer containing imidazole. IMAC purification of His-tagged protein is thought to be both affordable and relatively simple with potential for commercial scale, although the position of the His-tag could have an impact on the function and stability

of the target protein (Hu et al., 1999, Kuo and Chase, 2011, Park et al., 2015, Booth et al., 2018). Thus, optimising the His-tag position and length for IMAC purification is required to enhance protein expression and recovery of purified protein.

IMAC was used to purify the pEAQ-HT-H9-His-tag, pEAQ-HT-H5-His-tag, and pEAQ-HT-H2-His-tag proteins. The expression levels of pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag proteins were high, but the decreased amount of proteins detected in the elutions agreed with Mortimer et al. (2012). It is thought that the His-tag was not accessible enough to purify and recover all the proteins. Although the expression of the pEAQ-HT-H2-His-tag protein was low, possibly because of the lack of codon optimisation, the elution contained a good quantity of purified proteins. Comparison of the codon optimisation effect on protein expression should be done using the same construct to be more accurate. Adding a restriction site between the construct and the His-tag resulted in a good amount of proteins detected in the elutions, as in the case of pEAQ-HT-H2-His-tag proteins, pEAQ-HT-H9-SUMO-tag, and pEAQ-HT-H5-SUMO-tag recombinant proteins. The results show that codon optimisation might be essential to obtain a good yield of the target protein by enhancing the translation of the target proteins (Kwon et al., 2016).

VLPs have been successfully generated previously in *N. benthamiana* expressing avian $H5N1_{HA}$ (D'Aoust et al., 2008, Landry et al., 2010), H6N2 (Smith et al., 2020), and H7N9 (Pillet et al., 2015).

pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP were successfully expressed in this study using HA alone in *N. benthamiana*, and the expression was confirmed using western blot and TEM, and our results agree with (D'Aoust et al., 2008). Chen et al. (2007) suggested that M1 is vital for forming HA VLPs, and NA is not necessary because it is only essential for releasing the VLPs from the infected cells. D'Aoust et al. (2008) have managed to generate influenza VLPs from HA alone and found that M1 decreases the VLPs accumulation, and the cause of that is unknown. The secretory pathway directs HA to the plasma membrane, while M1 is a cytosolic protein that accumulates beneath the plasma membrane, and it could be that the HA and M cannot be accumulated at the budding site. D'Aoust et al. (2008) showed via TEM that influenza VLPs bud from the plasma membrane and accumulate at the indentations of the cell wall.

The TEM confirmed the presence of the pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP, measuring around 100 nm. The pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP were roughly spherical, and particle size was approximately 100 nm in diameter using TEM, similar to previous results regarding influenza VLPs produced in plants (D'Aoust et al., 2008, Lindsay et al., 2018). In another trial to generate H6 VLPs, the particles were pleomorphic and ranged from 70−100 nm in diameter (Smith et al., 2020).

The Bradford assay showed a difference between the pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP plant lysate and the negative control plant lysate. The pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP were partially purified, so it

is not accurate to depend on the Bradford assay results. Inactivated avian influenza vaccines contain 256 HAU per dose, so the HA test was done to measure the HAU in the plant lysate.

HA test was performed using concentrated pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP, and the results showed that the titre of pEAQ-HT-H5-VLP samples from different batches showed agglutination of the RBCs ranged from 128 to 256, while the titre of pEAQ-HT-H9-VLP samples using the modified VLP extraction ranged from 128 to 512. The modified VLP extraction buffer contained low salts that enhanced the amount of recovered VLPs and this could be because the high salts disrupt the assembly of the VLPs. Our VLP HA titre was slightly higher than those of (Phan et al., 2018), with a titre ranging from 64 to 128 with the H5 oligomers, and (Kanagarajan et al., 2012) had a similar titre of 128. Mortimer et al. (2012) had a lower HA titre 32 with both H5 full-length or truncated HA.

Our pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP are ready for testing the immunogenicity in mice and chickens.

4.1. Introduction

Recently, plants have been selected as a target for generating vaccines due to their low cost and high yield in plants (Peyret et al., 2021). Subunit vaccines are recombinant proteins that are poorly immunogenic, while VLPs are highly immunogenic and composed of repeats of structural proteins assembled for optimal identification by the APCs in the host (Fifis et al., 2004). VLPs can elicit both cellular and humoral immune responses, and experimental animals were used to test their immunogenicity (Monreal-Escalante et al., 2022).

4.1.1. Animal models

Several animal models have been developed for evaluating the efficacy of influenza vaccines and antiviral treatments, including mice, rats, guinea pigs, and ferrets (Ben-Yedidia and Arnon, 2005, Suguitan Jr et al., 2006, Alarcon et al., 2007, Ochi et al., 2008). For the study of many pathogens, mice are the preferred experimental model due to their readily manipulable genome, quick reproduction, ease of handling, low cost, and less variability. Mice and ferrets are the most often used animal models in influenza research among inbred mice.

Avian species express SA α-2, 3 Gal, while human influenza viruses bind to SA α-2,6 Gal (Ning et al., 2009). BALB/c and C57BL/6 are the most

frequently laboratory mice strains used (Margine and Krammer, 2014). BALB/c mice express the SA α-2,3 and SA α-2,6 Gal receptors on the lung, trachea, liver, cerebellum, spleen, and kidney, so the mouse is a good model for studying both human and avian viruses and testing vaccine immunogenicity (Ning et al., 2009). However, the IAV replicates in the lower respiratory tract of mice in contrast to humans; as a result, IAV is not transmitted between these species (Rodriguez et al., 2017). The mouse model is valuable for immunological studies because established and reliable assays are available. For example, cellular or humoral immune responses can be measured after vaccination using ELISpot or ELISA assays, respectively.

Chickens (*Gallus gallus domesticus*) are the natural host for avian influenza and are, therefore, often used in studies to develop vaccines or understand pathogenesis. This species is highly available and has a low cost. Additionally, chickens can be genetically altered (Hemmink et al., 2018). Using the chicken model has revealed additional knowledge on the pathogenesis of the influenza virus. Therefore, chicken is an ideal model for studying AIV virulence and vaccine efficacy (Nguyen et al., 2021).

4.1.2. ELISA

ELISA for detecting specific antibodies against AIV in poultry has been validated for use in the veterinary sector (WOAH, 2021). These usually use conserved epitopes on matrix protein or nucleoprotein conserved between all IAVs, as capture antigens. Antigen capture ELISA kits are inexpensive

and can detect IAV-binding antibodies, which is important for detecting infection and disease control. (WOAH, 2021). The World Organisation for Animal Health (WOAH) recommendations state that HI testing for subtyping to H5 and H7 must be performed after all positive results. Additionally, several ELISA antibody test kits for particular subtypes (e.g., H5 and H7) are now available (WOAH, 2021).

4.1.3. ELISpot

Cytokines are crucial immune response mediators that have been demonstrated to be essential for the immunological defence against microbial infection (Ochi et al., 2008). Interferon-gamma (IFN-*γ*) is a vital cytokine responsible for regulating the host immune response (Huang et al., 1993, Bouley et al., 1995). Most of the immune cells are covered by IFN-*γ* receptors. IFN-γ can affect T cell differentiation and activate Th1-type immune responses in CD4+ and CD8+T cells (Whitmire et al., 2005, Ochi et al., 2008). IFN upregulates class II major histocompatibility complex (MHC) production, which boosts the antigen presentation to CD4+ T cells by antigen-presenting cells (APCs), including DCs, macrophages, Langerhan's cells and B cells (Steeg et al., 1982, Wadee et al., 1995). Additionally, IFN-γ boosts class I MHC expression, enhancing the activation of CD8+ T antigen-specific cells (Maudsley and Morris, 1989, Thomas et al., 1998). The generation of host immunity against numerous viruses, including IAV, depends on Th1 cells (Karupiah et al., 1993, Katze et al., 2002, Halford et al., 2005). In addition, it is believed that IFN-γ production is a significant

factor in assessing vaccine effectiveness (Asanuma et al., 2000, Lee et al., 2003). IFN-*γ* measurement after infection and vaccination is crucial for understanding IAV. The IFN-*γ* ELISpot test is a technique for counting the number of T-cells that secrete a cytokine after being stimulated by a particular antigen (Smith et al., 2001). The number of spots rises in proportion to the intensity of the immune response. The IFN-ELISpot can evaluate a Th1 cell-mediated immune response indirectly. As a result, it helps assess how well a vaccine induces cell-mediated immunity. Two highly specific antibodies against separate epitopes on the same cytokine molecule are used in the ELISpot technique. A colourimetric reaction produces spots when a soluble substrate is broken down, producing an insoluble precipitate at the reaction site. The number of cytokine-producing T cells may be determined directly by counting the number of dots (Akache and McCluskie, 2021).

4.1.4. Pseudotyping

Pseudotype viruses (PV) are non-replicative viral particles expressing the target surface proteins of a specific virus and a unique gene as a core genetic material, e.g., a reporter gene, reducing the need for biosafety when investigating the pathogen (Garcia and Lai, 2011). They also provide an adaptable platform since it is possible to create particles with various surface proteins from newly emerging and re-emerging viruses (Sawoo et al., 2014, Nie et al., 2020). PVs also have several applications in the measurement of virus-neutralising antibodies. The selective usage of codons is a key factor

for obtaining optimal expression of recombinant proteins since many organisms have a preferred codon among the multiple codons that generate the same amino acid (Wang et al., 2012). HA PV usage is reported to be highly effective for identifying neutralising antibodies in the pseudotype virus neutralisation test (Wang et al., 2008, Alberini et al., 2009, Du et al., 2010, Sawoo et al., 2014). The neutralisation experiment involves combining the PVs with host serum containing neutralising antibodies against the HA, which will bind with the glycoprotein, cell fusion, and entry, preventing the reporter gene from being transferred (Alberini et al., 2009, Delgadillo-Gutiérrez et al., 2022).

Retroviral, lentiviral-based on human immunodeficiency virus core (HIV), and murine leukaemia virus are widely used in pseudotyping. Retroviral and lentiviral systems are extensively used to generate influenza pseudotypes (Carnell et al., 2015, Ferrara et al., 2021). Retroviral pseudotypes expressing IAV HA are utilised as safe surrogates to mimic the structure and surface HPAIVs as H5 to study the biological processes induced by the envelope proteins originating from these viruses for virus diagnosis and vaccine (Garcia and Lai, 2011, Sawoo et al., 2014, Del Rosario et al., 2021, Delgadillo-Gutiérrez et al., 2022).

This chapter describes the efficacy testing of H9 and H5 VLPs transiently produced in *Nicotiana benthamiana* (Chapter 3). Each VLP was formulated with an oil-emulsion adjuvant, and HA-specific antibody responses were determined using ELISA. Furthermore, sera were tested for their capacity to

neutralise heterologous PVs effectively. In addition, cellular immune responses were evaluated using ELISpot.

4.2. Material and methods

4.2.1. Post-vaccination sera

Sera were collected from chickens vaccinated with recombinant IAV with the HA and NA of A/Chicken/Pakistan/UDL01/08 with PR8 internal genes (H9N2) or A/Chicken/Cheboksary/854/2018 (H5N8) A/Puerto Rico/8/1934 (H1N1) with internal genes. Mouse-adapted H1N1 IAV, which causes serious infection in mice, is called PR8. The sera were kindly provided by Prof Munir Iqbal, Head of Avian Influenza Group, The Pirbright Institute.

4.2.2. Animal studies

The mouse trial was performed at the Life Science Group company, UK, and ethical approval was obtained (Project Licence PBFC8274D). Four BALB/c mice were used at five weeks old, with two mice used for each VLP (pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP). Each dose contained 480 haemagglutinating units (HAU) of VLPs (Chapter 3, section 3.3.4). TiterMax Gold adjuvant was mixed in equal amounts with the VLPs, around 0.18 ml each. Mice were immunised using the subcutaneous route with three doses at the two-week interval and were bled and sera collected after the third immunisation and the final bleed (Figure 4.1).

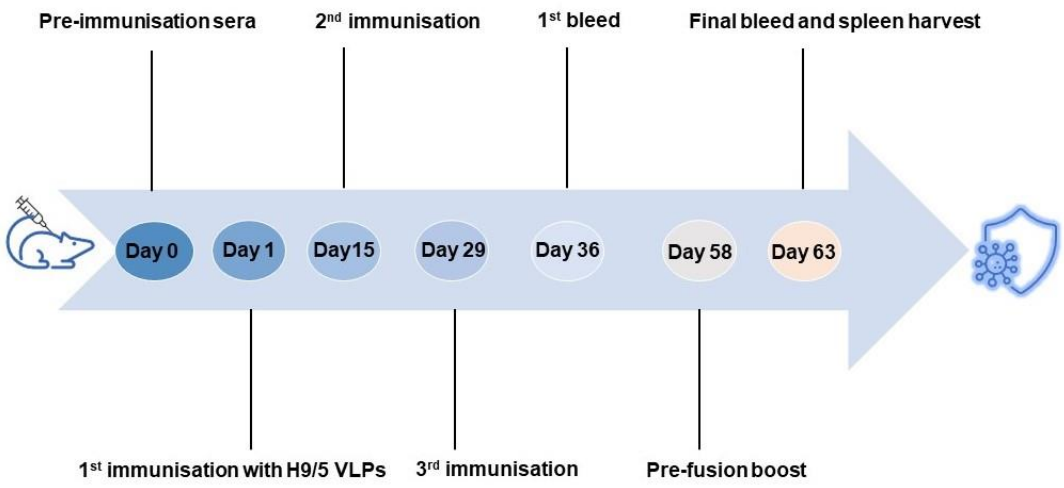


Figure 4.1. Demonstration of the mouse immunisation experimental design.

The chicken immunisation trial was performed kindly by Prof Munir Iqbal, Head of Avian Influenza Group, The Pirbright Institute. Six specific pathogen-free (SPF) Valo chicks (eggs from Valo Biomedia) at seven days old were used for an immunisation trial for the pEAQ-HT-H5-VLP with one dose of 0.1 ml containing 1024 HAU mixed with an equal amount of Montanide ISA 71 VG adjuvant (Seppic company). Chicks were bled every week up to six weeks post-immunisation (Figure 4.2).

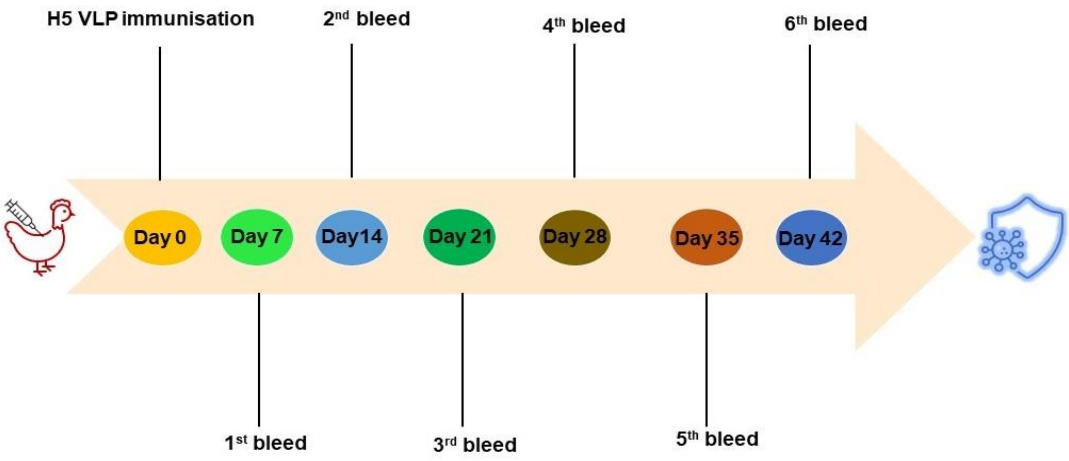


Figure 4.2. Demonstration of the chicken immunisation experimental design.

4.2.3. ELISA

The pEAQ-HT-H9-VLP, pEAQ-HT-H5-VLP, pEAQ-HT-H9-His-tag, and pEAQ-HT-H5-His-tag proteins were added to each well of a Nunc™ 96-well flat bottom plate (Thermo Fisher, UK) at a concentration of 2 µg/well in 100 µl of the coating buffer (Appendix D). The plates were incubated at 4°C overnight. The plates were then washed three times with 200 µl PBS-T (Appendix D) and PBS. The blocking buffer (Appendix D) was added to the wells at 200 µl/well and left at room temperature for one hour before washing. The plates were left at room temperature for two hours before washing as before. The secondary HRP-linked goat anti-chicken IgY(H+L) antibody (catalogue number 6100-05, SouthernBiotech, USA) or anti-mouse HRP-linked IgG Antibody (catalogue number 7076S, Cell Signaling Technology, Netherlands) was diluted in the blocking buffer and 100 µl/well added. The primary and secondary antibody concentration was optimised

and specified in the results for each experiment. The plates were left for one hour at room temperature. The plates were then washed three times with PBS-T and PBS. The TMB substrate (Thermo Scientific, UK) was added at 100 µl/well and incubated for 10 minutes; then, 100 µl/well 2M sulphuric acid was added. A Varioskan microplate reader was used to measure the absorbance at 450 nm.

4.2.4. ELISpot

ELISpot assay was performed using mice splenocytes from spleens collected at the end of the mouse immunisation trial. First, First, Roswell Park Memorial Institute (RPMI) 1640 media was prepared (Appendix D), and then the spleens were squeezed using a syringe containing 20 ml of RPMI media to flush the cells from the spleens into the precoated ELISpot Plus: Mouse IFN-γ (ALP) 96-well plates (code 3321-4APT-2, Mabtech, UK) using the media, and the cell suspension was centrifuged at 300 g for 10 minutes. The cell pellet was resuspended in 10 ml of RPMI medium containing 50 µM of 2-mercaptoethanol, and the cell suspension concentration was determined by counting using a haemocytometer (typically around $5x10^6$ cells/ml). The plates were incubated for 30 minutes at room temperature with RPMI media before the media was discarded. Then, 100 µl per well of spleen cells was added. Different concentrations of the H5 or H9 VLPs (0.1 µg/ml to 2.3 µg/ml) were prepared in RPMI, and 100 µl of these VLPs was added to duplicate wells containing splenocytes from pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP immunised mice, as appropriate. Concanavalin A (con A)

(500X) (reference 00-4978-93, Invitrogen, Thermo Fisher Scientific, UK) 0.5 µl per ml of RPMI media (final concentration 0.25X) containing 50 µM of 2-mercaptoethanol was used as a positive control. Two negative controls were used, one of them was the well-containing splenocytes and negative control lysate (plants infiltrated with empty pEAQ-HT vector), and the other was the cells and media only. The plates were incubated at 37°C for 48 hours in a 5% CO_2 incubator. Then, the cells were flicked off the plates, and the experiment was developed according to ELISpot Plus: Mouse IFN-γ (ALP) kits (code 3321-4APT-2, Mabtech, UK) guidelines. The spots were counted using an ImmunoSpot® Analyzer (Figure 4.3).

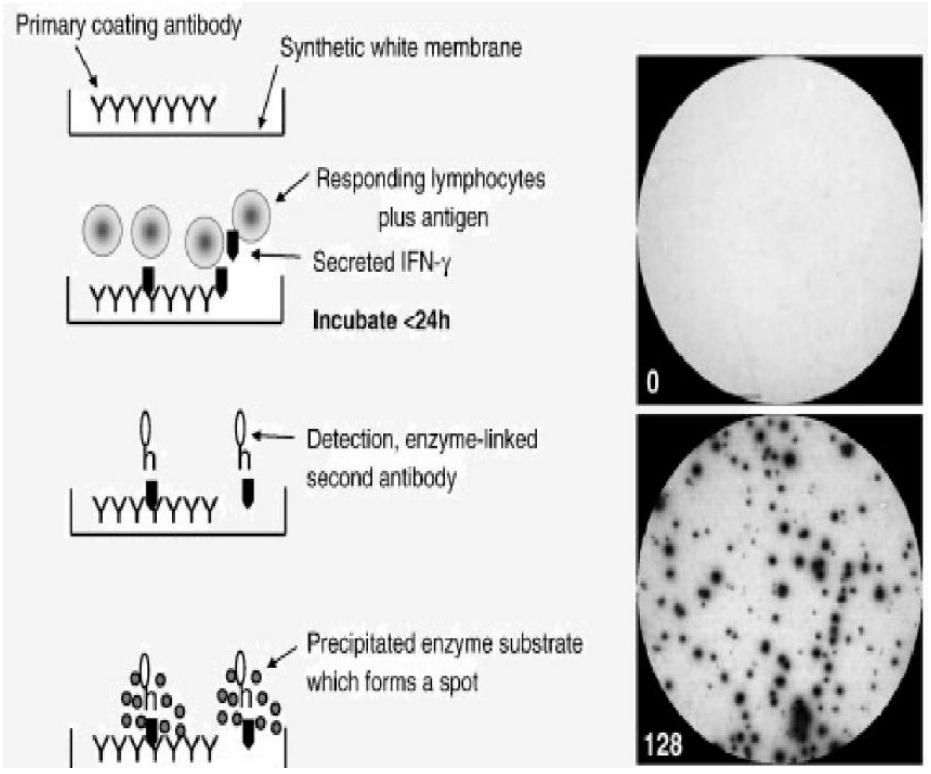


Figure 4.3. Demonstration of IFN-γ ELISpot steps adapted from Augustine and Hricik (2012).

4.2.5. Cell culture

Human embryonic kidney (HEK) 293T cells were used as producer and target cells in PV production and titration. A single aliquot of the 293T cells was initially thawed from liquid nitrogen by immersion in a 37°C water bath for 1–2 minutes and resuspended in Dulbecco's Modified Eagle Medium supplemented with 1% L-glutamine, 100 U penicillin, and streptomycin (1µg/ml) and 10% fetal calf serum, also referred to as D-10 (Appendix D). The cells were centrifuged at 300g for 5 minutes, the D-10 and freezing media were removed, and cells were resuspended in fresh D-10. Cells were cultured in T75 filtered Nunc™ EasYFlask™ (Thermo Scientific™ UK) at 37°C in 5% CO2 and sub-cultured by removing D-10, washing with PBS before detaching the cells from the T75 flask with 3ml of 1x trypsin solution, incubated at 37°C for 5 minutes, and resuspended in fresh D-10 media. Seeding density was determined based on how quickly the cells grew and when the cells were to be used, ranging from $1x10^4 - 1x10^5$ cells/ml.

4.2.5.1. Pseudotyped virus assays

4.2.5.1.1. Plasmids

This study used the HIV-1 lentivirus packaging construct psPAX2, a second-generation plasmid encoding the gag-pol genes. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260 ; http://n2t.net/addgene:12260 ; RRID:Addgene_12260). The pCAGGS-HAT plasmid was used to express the HAT protease, facilitating the cleavage of influenza HA (Figure 4.4). pCAGGS-HAT was kindly provided by Prof Eva Böttcher-Friebertshäuser

(Institute of Virology, Philipps University Marburg, Germany) (Böttcher-Friebertshäuser et al., 2010). The pCSFLW lentiviral vector expressing firefly luciferase was also used to provide a robust signal for quantifying the light emitted when target proteins are expressed. pCSFLW was provided to us by Dr Simon Scott, University of Kent (Addgene plasmid # 12158 ; http://n2t.net/addgene:12158 ; RRID:Addgene_12158). The HA and the NA genes were first cloned into a mammalian expression vector pI.18. H9 (A/Hong Kong/1073/99) (H9N2) in pI.18 were kindly received from Prof. Nigel Temperton and Dr Simon Scott, Viral Pseudotype Unit, Medway School of Pharmacy, Kent, while NA (N2) was PCR-amplified from A/canine/Illinois/41915/2015 (H3N2). The H5 in pI.18 was PCR-amplified from A/Vietnam/1194/2004(H5N1), while NA (N8) was amplified from A/equine/Newmarket/5/2003 (H3N8) (Table 4.1). Recombinant neuraminidase from *Clostridium perfringens* (catalogue number: 9001-67-6, Sigma, UK) was used to compare the effect of the exogenous and endogenous NA on the PV titre.

Table 4.1. Details of the viruses and plasmids used in the pseudotyped virus generation.

Plasmids (pI.18)	**Virus**	**Accession number**
-H9$_{HA}$	Synthesized from A/Hong Kong/1073/99 (H9N2)	AJ404626
- H5$_{HA}$	Amplified from A/Vietnam/1194/2004(H5N1)	AAT73273

- $N2_{NA}$	Amplified from A/canine/Illinois/41915/2015 (H3N2)	MF173120
- $N8_{NA}$	Amplified from A/equine/Newmarket/5/2003 (H3N8)	FJ375224

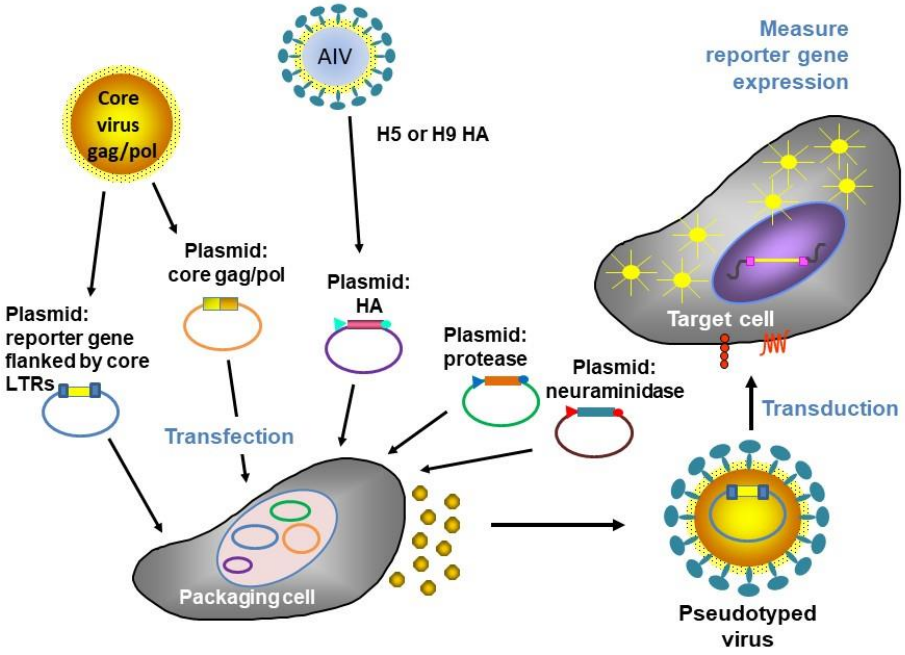


Figure 4.4. Demonstration of PV generation process modified from Scott et al. (2012).

4.2.5.1.2. Pseudotyped virus production

All pseudotyped viruses (PVs) were produced in 6-well plates using cultured 293T cells, seeded at a density of $3x10^5$/well, and transfected at approximately 60 –70% confluency (typically 2 days after seeding). Plasmid concentration was measured using the nanodrop™ 8000 spectrophotometer (Thermo Fisher Scientific, UK), and quantities were calculated accordingly

(Table 4.2). Vesicular stomatitis virus glycoprotein G (VSV-G) was a positive control for the transfection efficiency because of its ability to infect a wide range of cells. A delta envelope (ΔEnv) negative control only comprised the core and reporter plasmids. Five plasmids were added to the cells in the well. Into each well was aliquoted 100µl of OptiMEM™ (reference: 11058021, Thermo Fisher Scientific, UK) with 7 µl of lipofectamine™ 2000 (reference number: 11668-027 Thermo Fisher Scientific, UK), and a second aliquot containing 100µl of OptiMEM™ and the required DNA with a maximum capacity of 2500 ng DNA per well. The two aliquots were premixed and incubated at room temperature for 15 minutes before adding to the well, gently dropping steadily around the well before gently rocking the plate to ensure complete coverage of the cell monolayer. The supernatant containing the PVs was harvested after 48 hours of incubation at 5% CO_2 and 37°C, and PVs were filtered using a 0.45 µm syringe filter and stored in aliquots at -80°C until further use.

Table 4.2. Transfection components and quantities for PV production

Transfection components	**6-well culture plate (per well)**		
Plasmids	HA PV	HA+NA PV	VSV-G PV
pI.18-HA	500 ng	500 ng	-
pI.18-NA	Exogenous NA	125 ng	-
psPAX2 - core gag/pol (core)	500 ng	500 ng	500 ng

pCSFLW (reporter)	1µg	1µg	1µg
pCAGGS-HAT	125 ng	125 ng	-
VSV-G	-	-	500 ng
Transfection reagent			
Lipofectamine™ 2000		7µl per PV	
OptiMEM™		100µl x 2	

4.2.5.2. Pseudotyped-virus titration

Two methods for PV titration (PVT) were compared. The first (method A) was performed according to Ferrara and Temperton (2018). Using a 96-well flat-bottomed cell culture plate, 100 µl of neat PV supernatant was added in duplicate to row H. A total of 50 µl of D-10 medium was added to all other wells, and 50 µl of PV supernatant was serially diluted from H to A, discarding the final 50 µl. The end column (12) was reserved for a cell-only control. 293T cells were added to all wells at $1x10^4$ cells in a volume of 50 µl. The plates were incubated at 37°C with 5% CO_2 for 48 hours. Once complete, the contents of the plates were removed, and 50 µl Promega Steady-Glo® (Promega, Southampton UK) was added to all wells. After allowing the cells to lyse for 10 minutes at room temperature, the plate was read using a Varioskan microplate reader, and the results were reported in relative luminescent units (RLU)/ml. The titre was calculated by multiplying the RLU reading by the dilution factors appropriate for each dilution, e.g. 50 µl x 20 = 1000 µl = 1ml. Therefore: RLU reading x 20 = RLU/ml value. The following multiplication factors were applied to the remaining values ranging

from least to most titrated (40/80/160/320/640/1280/2560). The mean of all the RLU/ml values was calculated to give an overall titre. Inhibitory maximal concentration (IC50 or IC90), the serum concentration needed for neutralisation of 50% or 90% of the pseudotypes, was calculated according to the method detailed by Ferrara and Temperton (2018) using GraphPad Prism 9 software.

Method B was described by Nie et al. (2020). As for method A, a 96-well flat-bottom culture plate was used to titrate the pseudotyped virus. The outermost wells were used as an evaporation barrier with 250 µl of sterilized water per well. Complete DMEM (135 µl) was added to wells B2–G2. Complete DMEM (100 µl) was added into the remaining wells (B3–G11). Each PV was added in (15 µl) per well to wells B2–G2 to make an initial dilution of 1:10. The serial dilution was started by transferring 50 µl of solution from wells B2–G2 to wells B3–G3 and then transferring 50 µl transferred from wells B3–G3 to wells B4–G4. The transfer was repeated to wells B10–G10. After mixing, 50 µl of solution were aspirated from each well in column 10 and discarded. The dilution step does not involve column 11 (B11–G11), which will be used as the cell control (CC). 293T cells were added to the 60 inner wells (B2–G11) at 2 $\times 10^5$ cells/ml in 100 µl. The titration plate was incubated in the 5% CO_2, 37°C incubator for 20–28 h. Once complete, the contents of the plates were removed, and 50 µl Promega Steady-Glo® (Promega, Southampton UK) was added to all wells. After allowing the cells to lyse for 10 minutes at room temperature, the plate was

read using a Varioskan microplate reader, and the results were reported in relative luminescent units (RLU)/ml. The results were added to the Excel spreadsheet provided in the supplementary materials (Nie et al., 2020), which was used to calculate the tissue culture infective dose$_{50}$ ($TCID_{50}$)/ml for use in the Pseudotyped virus neutralisation test (PVNT) assay.

4.2.5.3. Pseudotyped virus neutralisation test

Method A (Ferrara and Temperton, 2018) was used to titrate the neutralising antibodies in the post-vaccination chicken sera and immunised mice sera. A total of 90 µl of D-10 media was added to all wells except the first well in triplicate rows, including six-virus control wells and six-cell control wells. A total of 5 µl of H5 or H9 antiserum and 95 µl of D-10 were added to the first well of triplicate rows. A ten-fold serial dilution was performed by sequentially removing 10 µl from the first well and transferring it to the next well, pipetting several times to mix, and discarding 10 µl from the final wells. The plate was spun for 1 minute at 500 g. PV stocks were prepared as necessary and 50 µl containing $1x10^6$ RLU was added to each well. The plate was spun for 1 minute at 500 g in Thermo Scientific Megafuge 16 Heraeus centrifuge. The plates were left for 1 hour at 37°C and 5% CO_2. Before the end of the incubation period, 293T cells were trypsinised and counted using a haemocytometer. A total of 50 µl per well containing $1x10^4$ cells were added to all wells. The plate was spun for 1 minute at 500 g. The plates were incubated for 48 hours at 37°C and 5% CO_2. The supernatant was removed from all wells, and the cells were harvested by adding 50 µl/well of Steady

Glo reagent. The cells were left to lyse for 10 minutes at room temperature, and then the luminescence was measured using a Varioskan microplate reader (Thermo ScientificVarioskan® Flash).

Method B (Nie et al., 2020) was used to test the immunised chicken sera. Serum samples were inactivated at 56°C for 30 minutes to destroy non-specific inhibitors. A 96-well flat-bottom culture plate was used to perform the PVNT. The outermost wells were used as an evaporation barrier with 250 µl of sterilized water per well. A complete DMEM medium (150 µl/well) was added in column 2 (B2–G2), which is used as the cell control (CC). To the wells of column 3 (B3–G3) was added 100 µl/well of complete DMEM medium designated as the virus control (VC). Complete DMEM (142.5 µl/ well) was added to the remaining wells of row B (B4–B11), the initial dilution row. Complete DMEM (100 µl/well) was added to the remaining wells (C4–G11). Serum samples (7.5 µl) were added in duplicate to row B (B4–B11) in the following order: sample 1, wells B4–B5; sample 2, wells B6–B7; sample 3, wells B8–B9; sample 4, wells B10–B11. The samples were mixed in row B 50 µl from each well and transferred from rows B and C. The mixing and transfer were sequentially repeated to row G (threefold serial dilutions). After the final transfer, 50 µl liquid was discarded from the wells in the final row of columns (wells G4–G11). The PV was diluted to 1.3 $x10^4$ $TCID_{50}$/ml in complete DMEM in a room-temperature water bath. The diluted PV (50 µl) was added to each well in columns 3–11. The plates were incubated in a 5% CO_2, 37°C incubator for 45–90 min. After the incubation, 293T cells were added to the 60 inner wells (B2–G11) at 2×10^5 cells/ml in 100 µl. The plate

was incubated in the 5% CO_2, 37°C incubator for 20–28 h. Once complete, the contents of the plates were removed, and 50 µl Promega Steady-Glo® (Promega, Southampton UK) was added to all wells. After allowing the cells to lyse for 10 minutes at room temperature, the plate was read using a Varioskan microplate reader. The results were added to the Excel spreadsheet provided in the supplementary materials (Nie et al., 2020), and the neutralisation titre was calculated for each serum sample.

4.3. Results

4.3.1. Testing the epitope integrity of recombinant proteins and VLPs

4.3.1.1. ELISA assay using post-vaccination sera

An ELISA test confirmed that pEAQ-HT-H9-VLP, pEAQ-HT-H5-VLP, pEAQ-HT-H9-His-tag, and pEAQ-HT-H5-His-tag proteins were recognised using post-vaccination H9 or H5 chicken sera (Figure 4.5). The sera were used at a concentration of 1:1000, and the secondary anti-chicken antibody at a concentration of 1:2000 for all samples, including the negative controls. The pEAQ-HT-H9-VLP1 extracted with a high salt buffer gave a measurable OD of 0.67; however, the pEAQ-HT-H9-VLP2 extraction using low salts buffer gave a higher OD of 2.78. A low salt extraction buffer is important for the recovery of the VLPs. The pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP had a higher OD of 2.78 and 0.91, respectively, compared to the pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag proteins and H9 purified elutions (OD 0.82, 0.79 and 0.60, respectively). The negative control lysate treated with H9 and H5 sera showed negligible OD (0.13).

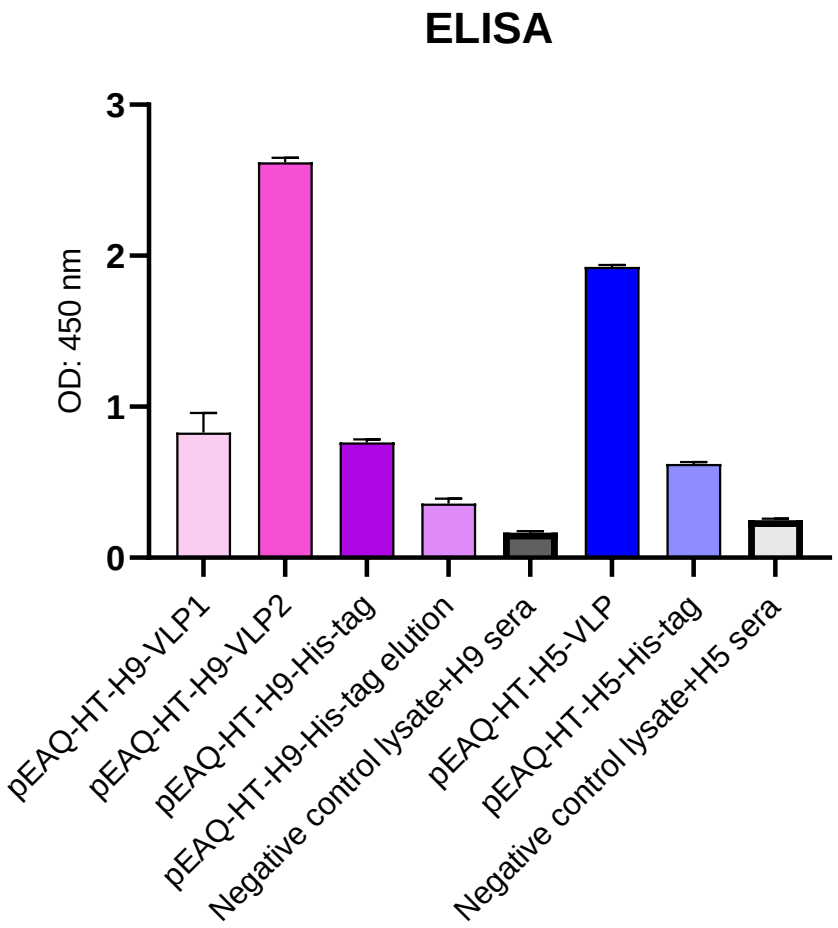


Figure 4.5. ELISA results for pEAQ-HT-H9-VLP, pEAQ-HT-H5-VLP, pEAQ-HT-H9-His-tag, and pEAQ-HT-H5-His-tag proteins using post-vaccination sera. The error bars represent the standard error of the mean (SEM) of the duplicate for each sample.

4.3.1.2. PVT using the post-vaccination sera

Method A was used to measure the titre of the H9 and H5 pseudotypes to determine the $TCID_{50}$ needed for PVNT. The RLU/ml calculated for the H9 PVs generated with endogenous NA (i.e. plasmids expressing N2 or N8 NA) were similar by around 1.8×10^7 and $8.2 \times 10^{6,}$ respectively were higher than

the H9 PV generated using exogenous NA (neuraminidase from Clostridium perfringens) of around 5.2 x 10^3 RLU/ml. The RLU/ml calculated for the H5 PV (generated by co-transfection with an N8 NA) was around 3.6 x 10^8, nearly similar to the VSV-G positive control (3.3 x 10^8). The ΔEnv showed 6.7x 10^2 RLU/ml (Figure 4.6).

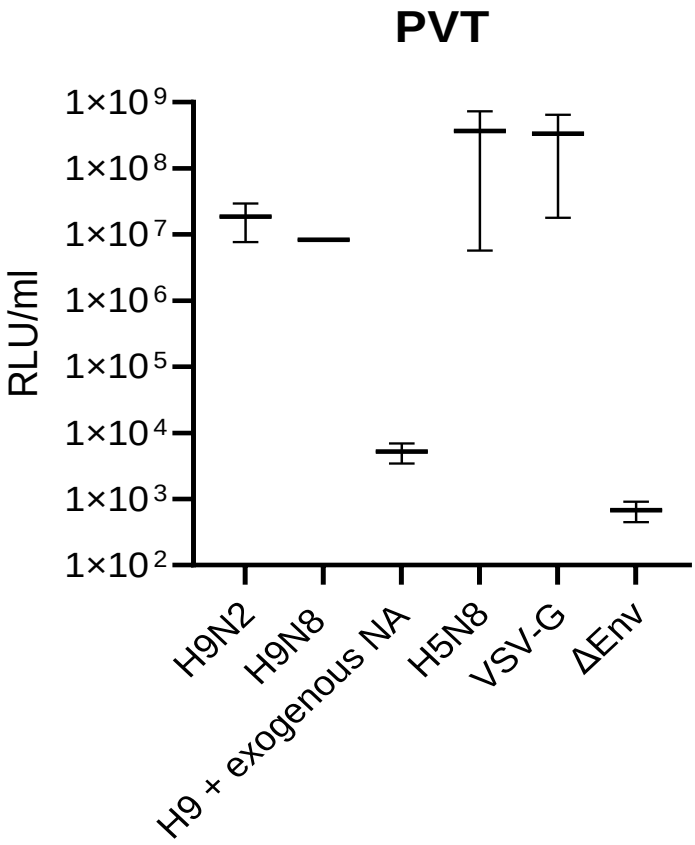


Figure 4.6. PVT of the H9 and H5 pseudotypes. The PV titre was determined for the H9 pseudotype generated using NA2 or NA8, or exogenous NA. H5 pseudotype was generated using NA8. VSV-G was used as a positive control, while ΔEnv PV was used as a negative control. The error bars represent the SEM of the biological duplicate replicates for each sample.

4.3.1.3. PVNT using the post-vaccination sera

Method A to measure the ability of post-vaccination chicken sera to neutralise PV entry and validate the PV assay. Both sera showed a high percentage of neutralisation of the heterologous PV, up to 100% (Figure 4.7). IC50 and IC90 for H9 sera were 144.97 and 30.23, respectively, while IC50 and IC90 for H5 sera were 6.1 and 3.4.

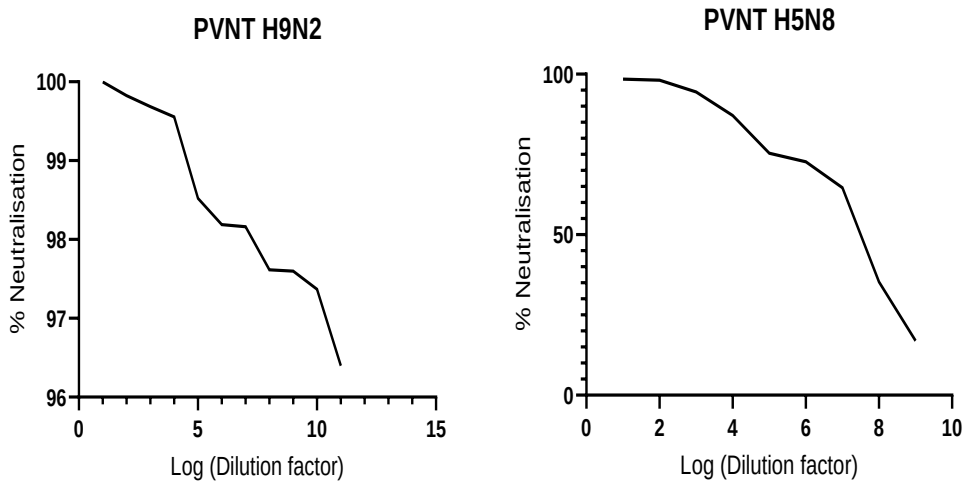


Figure 4.7. PVNT for H9 and H5 pseudotypes using post-vaccination chicken sera.

4.3.2. Mouse immunisation study

4.3.2.1. ELISA using mice sera

ELISA was used to screen sera from immunized mice (Figure 4.8). Mice sera were used at a concentration of 1:1000, and the secondary anti-mouse antibody at a concentration of 1:2000 for all samples, including the negative controls. The third immunisation sera and final bleed sera were mixed for each mouse to get sufficient volume for the assay. The results from each

mouse were very similar, and the graph presented the mean. H9 and H5 VLPs showed a high humoral immune response in the immunised mice with OD of 3.89 and 3.81, respectively. The pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP treated with pre-immunisation sera showed no antibodies (OD of around 0.09 and 0.06, respectively). The negative control lysate treated with H9 and H5 sera showed negligible OD (0.27 and 0.35, respectively).

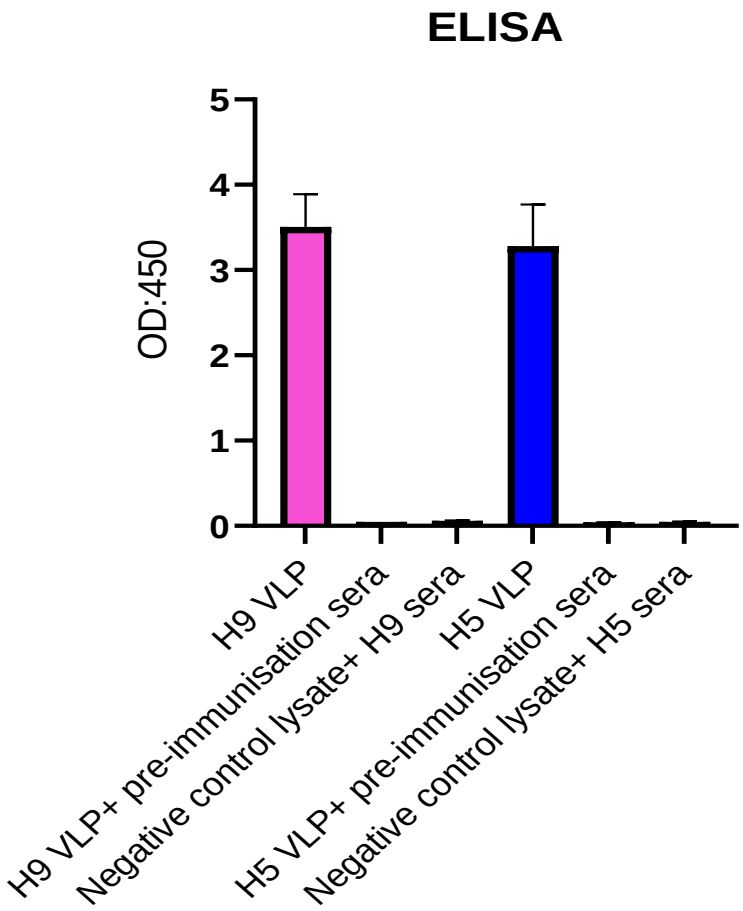


Figure 4.8. ELISA test for pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP using pooled immunised mice sera. The error bars represent the SEM of the triplicate for each sample.

4.3.2.2. PVNT using mice sera

Method A, PVNT, immunised mice sera showed a high percent of neutralisation of the H9N2 and H5N8 pseudotypes, up to 100% in the case of H9 sera and up to 98% in the case of H5 sera (Figure 4.9). The results revealed that immunisation of mice with either pEAQ-HT-H9-VLP or pEAQ-HT-H5-VLP stimulated a solid neutralising antibody against the H9 and H5 heterologous virus strain. IC50 and IC90 for H9 sera were 5.06 and 1.92, respectively, while IC50 and IC90 for H5 sera were 28.21 and 4.16. The H9 and H5 pre-Immunisation sera did not neutralise the H9 and H5 pseudotypes.

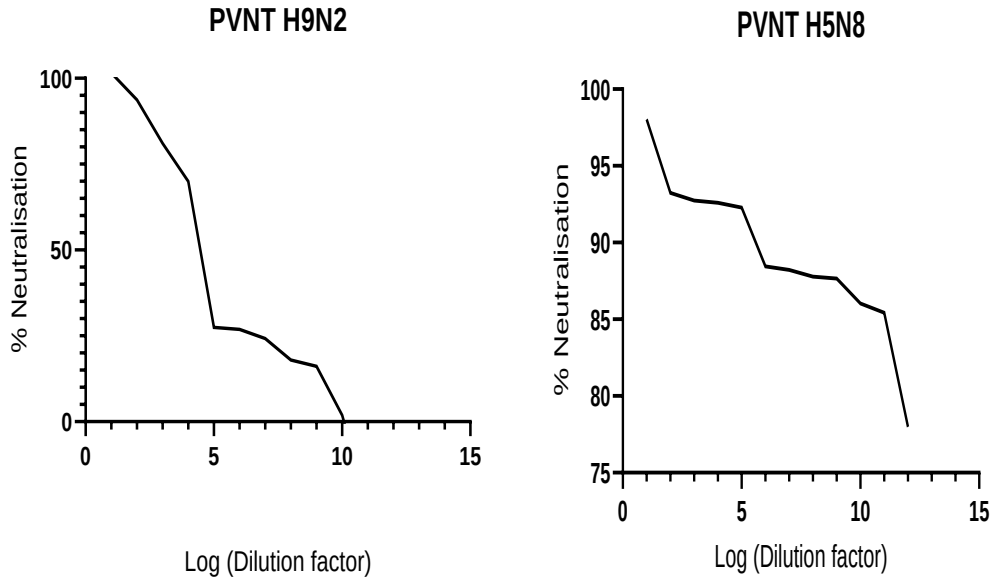


Figure 4.9. PVNT for H9 and H5 pseudotypes using immunised mice sera.

4.3.2.3. ELISpot

The ELISpot results showed a high number of spots using low concentrations of the VLPs (0.1−2.4 µg/ml) for restimulation compared to

the negative controls (Figures 4.10 − 4.11). The results revealed that a high number of interferon-γ secreting splenocytes were detected on restimulation of cells from immunised mice with pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP (297 and 301, respectively). The cell control only and the negative control lysate showed a negligible number of spots (6 and 42, respectively).

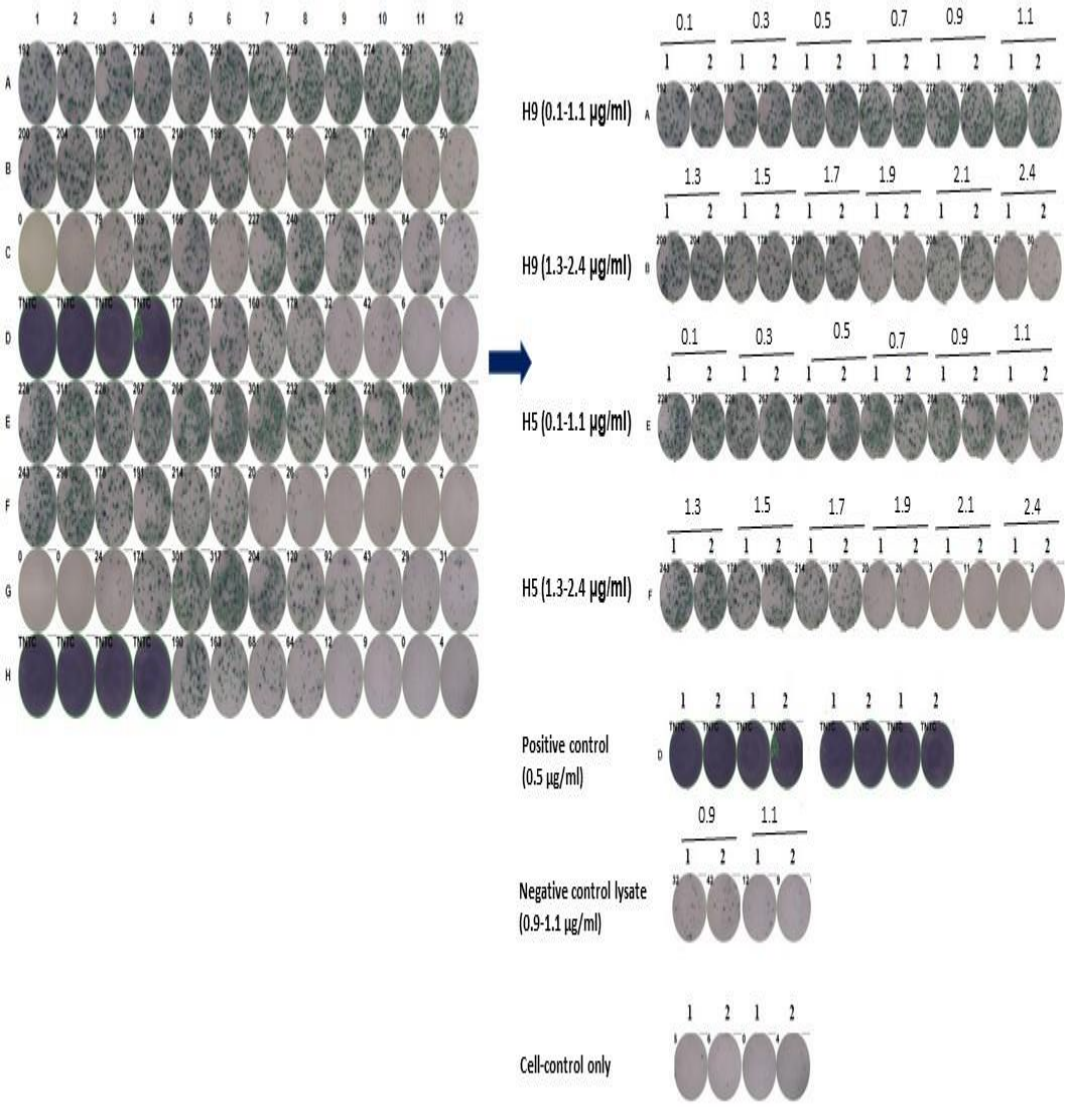
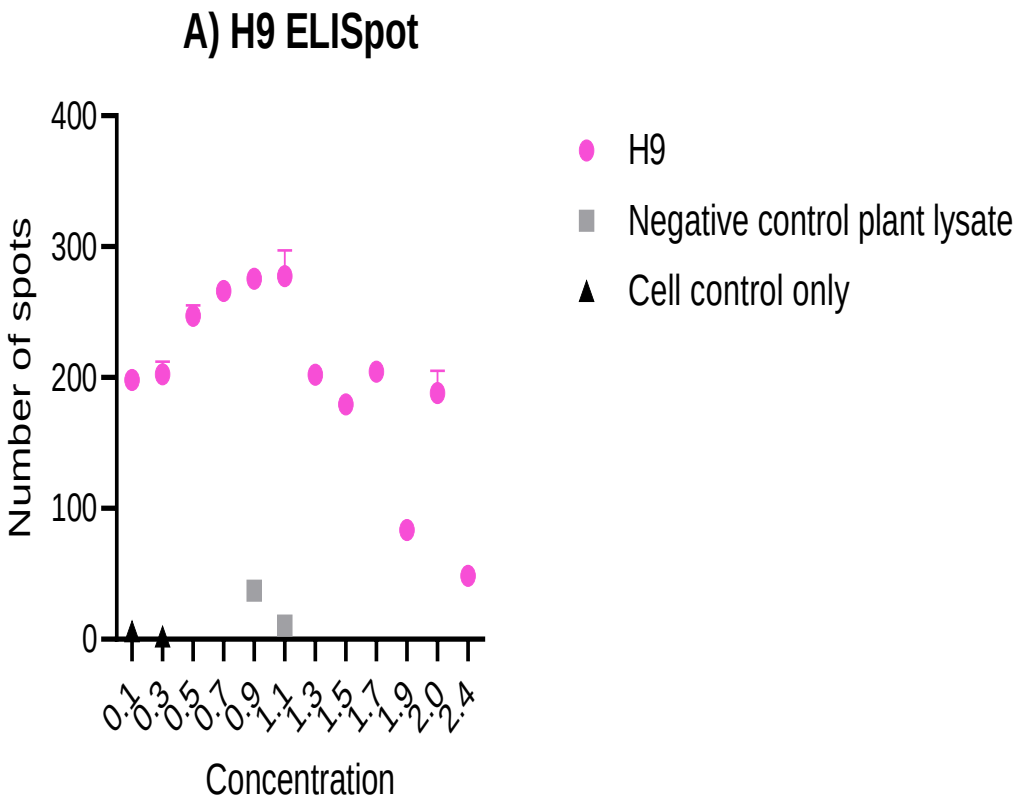


Figure 4.10. Plate design and results for interferon-γ ELISpot. Splenocytes from the immunized mice were seeded in 96-well plates precoated with an anti–interferon-γ antibody. T-cell responses were assessed after the

addition of a pEAQ-HT-H9-VLP (0.1–2.4 µg/ml) and pEAQ-HT-H5-VLP (0.1–2.4 µg/ml) or Concanavalin A (ConA) served as a positive control at 0.5 µg/ml. Additional controls were prepared (Cell control only or negative control plant lysate containing the empty pEAQ-HT at 0.9–1.1 µg/ml).



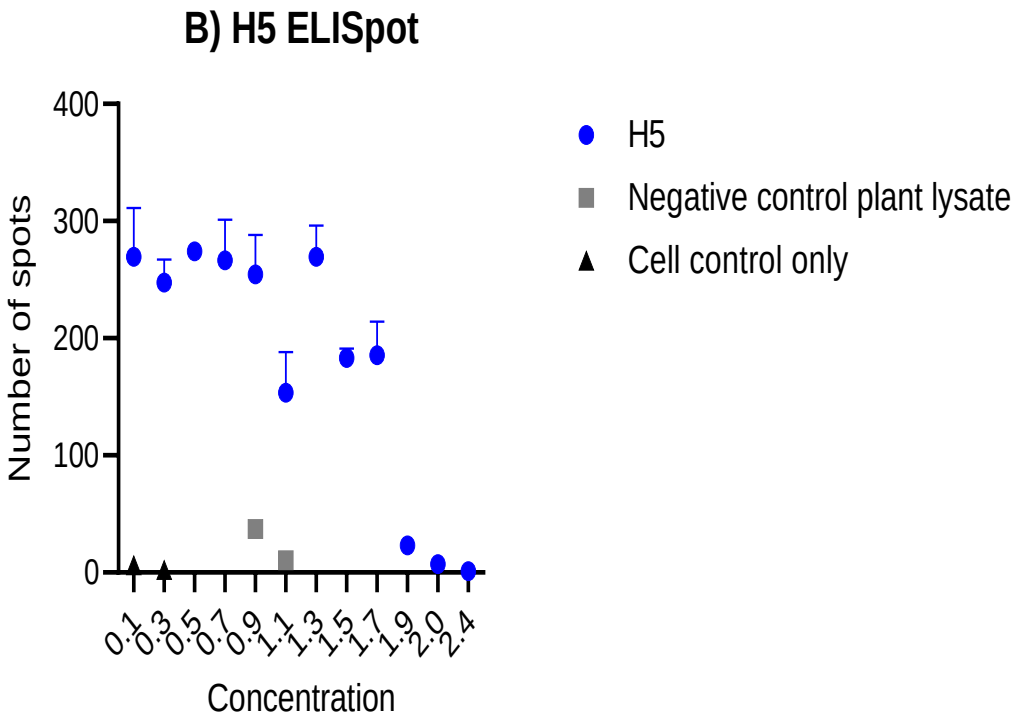


Figure 4.11. ELISpot results using spleens from immunised mice. Figures A and B represent ELISpot results for pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP. The error bars represent the SEM of the duplicate for each sample.

4.3.3. Chicken immunisation study

4.3.3.1. ELISA with chicken sera

ELISA test using immunised chicken sera confirmed that pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP were recognised using the immunised chicken sera (Figure 4.12). Chicken sera were used at a concentration of 1:2000, and the secondary anti-chicken antibody at a concentration of 1:3000 for all samples, including the negative controls. ELISA showed an OD of around 2.84 at day 7 for all chicks, then reached the highest value by day 14, 21, and 28 of

around 3.64, and decreased again until reaching the lowest value by the end of the trial at day 42 at around 1.94.

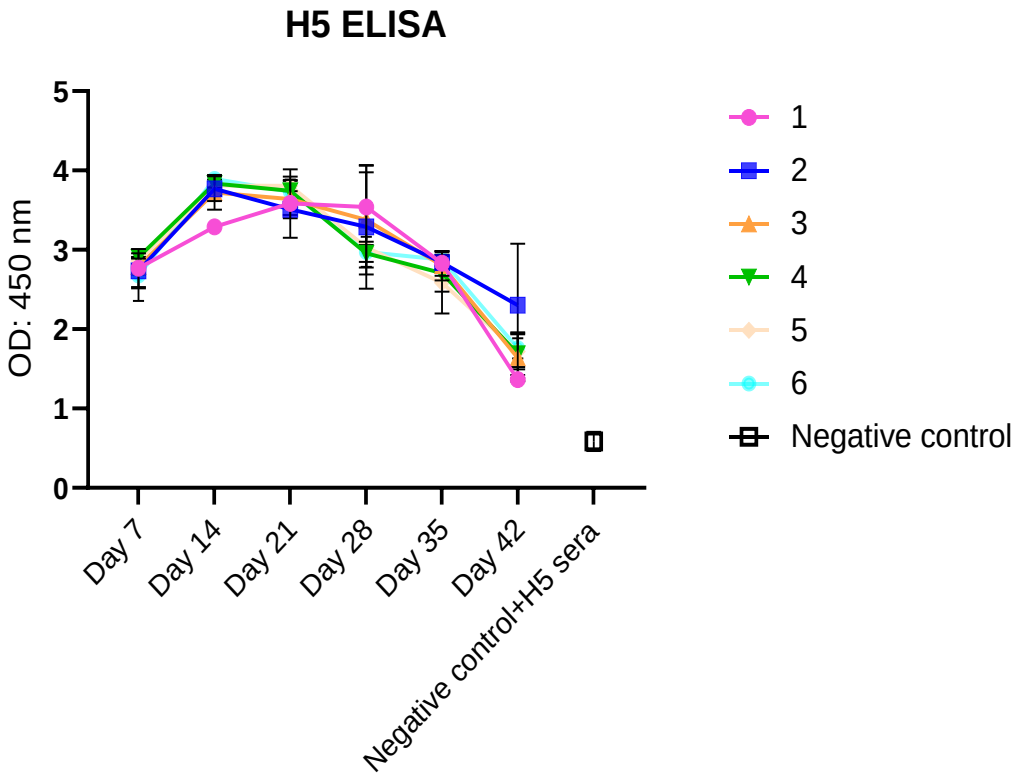
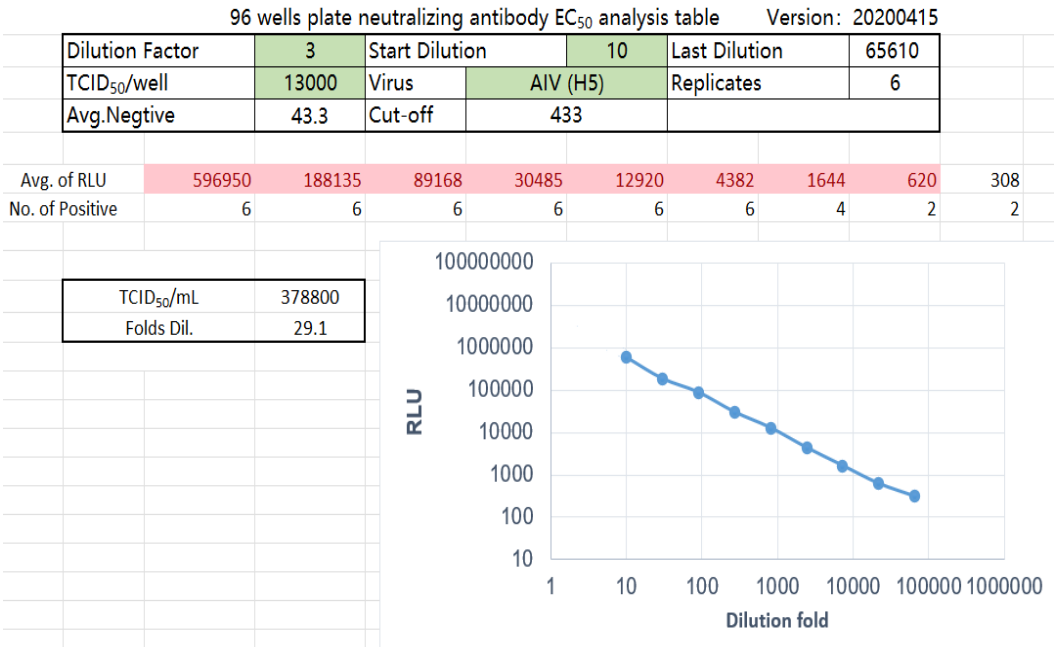


Figure 4.12. ELISA test for the pEAQ-HT-H5-VLP using immunised chicken sera. The error bars represent the SEM of the duplicate for each sample.

4.3.3.2. PVT using chicken sera

The H5 pseudotype was titrated using method B to determine the $TCID_{50}$/ml needed for PVNT. The $TCID_{50}$/ml of the H5 pseudotype was 378800 (Figure 4.13).



96 wells plate neutralizing antibody EC_{50} analysis table Version: 20200415

Dilution Factor	3	Start Dilution	10	Last Dilution	65610
$TCID_{50}$/well	13000	Virus	AIV (H5)	Replicates	6
Avg.Negtive	43.3	Cut-off	433		

Avg. of RLU	596950	188135	89168	30485	12920	4382	1644	620	308
No. of Positive	6	6	6	6	6	6	4	2	2

$TCID_{50}$/mL	378800
Folds Dil.	29.1

Figure 4.13. PVT of the H5 pseudotype.

4.3.3.3. PVNT using chicken sera

PVNT was performed with H5 immunised chicken sera and H5 PVs using method B. From the results, A low immune response was detected on day 7, reached the maximum by days 14 and 21, and decreased again until reaching the lowest value by the end of the trial at day 42 (Figure 4.14). The maximum neutralisation value was recorded on day 21 for chick 2 (Figure 4.15), with the highest neutralisation value up to 87%.

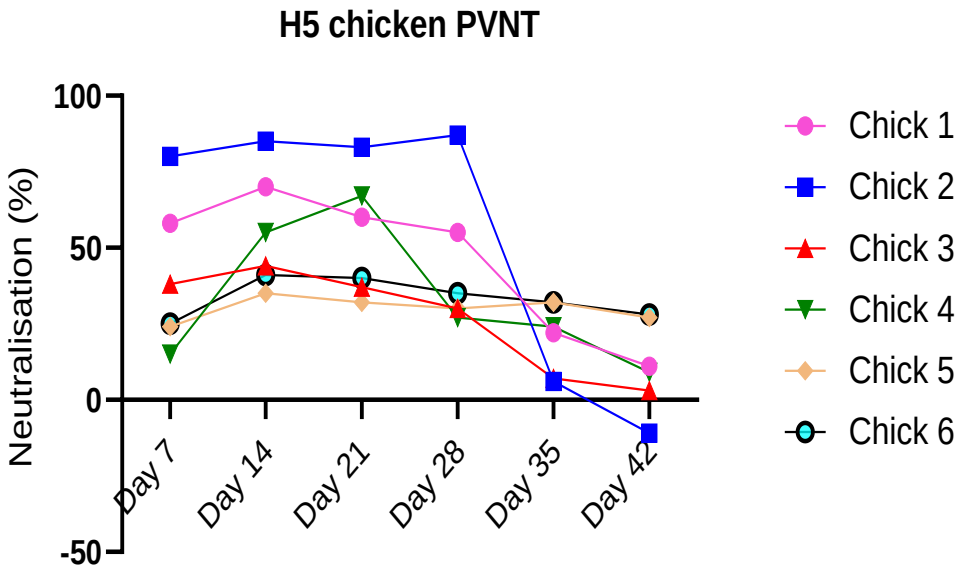


Figure 4.14. PVNT for the pEAQ-HT-H5-VLP using immunised chicken sera.

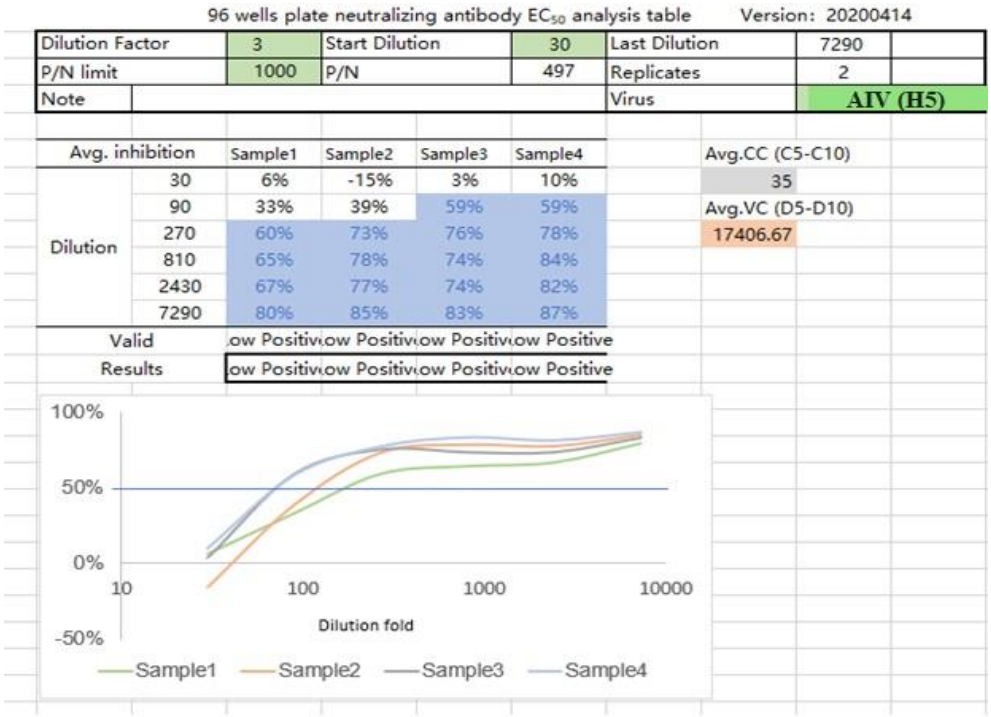


Figure 4.15. PVNT of the pEAQ-HT-H5-VLP using immunised chicken sera for chick 2 from day 7 to day 14. Sample 1: day 7, sample 2: day 14, sample 3: day 21, and sample 4: day 28.

4.4. Discussion

ELISA assay for VLPs was performed using sera from chickens experimentally vaccinated with viruses heterologous to our vaccine strain, and a high OD was detected compared to the His-tagged proteins. This suggests that the relevant HA epitopes are well presented on the surface of the VLPs and appropriately folded, which is why higher OD readings observed with VLPs compared with VLPs than the recombinant HA proteins are observed. In comparison, the recombinant proteins showed lower results than the VLPs that agree with (Tanguay and Killion, 1994, Ji and Forsthuber, 2016).

Medical research has long relied on the use of laboratory mice to assess the effectiveness and immunogenicity of vaccines (Coughlan, 2021). The ELISpot technique was adapted to detect the cellular immune response elicited after vaccination in mice which is very important, especially in the clinical trials of vaccines (Hutchings et al., 1989, Ji and Forsthuber, 2016). ELISpot technique can detect the cytokine-producing cells even if the secreted cytokine is below the detection limit by ELISA (Tanguay and Killion, 1994, Ji and Forsthuber, 2016). Mice have been frequently used as an animal model for testing plant-based recombinant proteins and vaccines against AIV (Spitsin et al., 2009, Mortimer et al., 2012, Major et al., 2015, Ceballo et al., 2017, Pua et al., 2017, Phan et al., 2018, Thi Pham et al., 2019).

IFN-γ ELISpot detects the cellular immunity elicited in mice after a vaccine trial. Our VLPs showed a high number of interferon-γ secreting splenocytes were detected on restimulation of cells from immunised mice with H9 and H5 VLPs through the ELISpot technique. In a study, mice immunised with plant recombinant H5 proteins showed a slightly lower response in ELISpot than our VLPs, while the same study showed a similar immune response in ELISA than our recombinant protein immune response (Wang et al., 2019). In another study, mice immunised with a plant recombinant H5 proteins showed a lower T-cells secreting splenocytes than our VLPs, while in the case of ELISA showed a low immune response similar to our recombinant protein results (Wang et al., 2020). Laddy et al. (2007) generated a DNA vaccine against H5N1, immunised mice with three doses, and measured the elicited immunity using ELISpot and ELISA. A high level of vaccine-specific cellular immunity was detected using ELISpot, but a poor humoral response was detected compared to our expected results for the DNA vaccine. Wang et al. (2019) generated a recombinant H5 protein in plants, used these proteins in the immunisation of mice with three doses, and detected a good cellular response but a low humoral response using ELISpot and ELISA, respectively.

In this study, immunogenicity testing of the pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP using immunised mice sera showed a specific humoral immune response in ELISA. Our results showed a specific immune response after the third dose in mice. Thi Pham et al. (2019) showed lower binding

antibodies in ELISA using immunised mice sera against plant-based H5 recombinant proteins than observed for our H5 VLP ELISA data. Phan et al. (2018) showed a slightly lower binding antibody response in ELISA using immunised mice sera collected after the third immunisation against plant-based H5 oligomers proteins than our H5 VLPs ELISA results. In contrast, mice were immunised with two doses of plant-based H5 recombinant proteins, and the antibody immune response started to be detected after the second immunisation compared to the negative control group (Major et al., 2015). In another study, mice were immunised with the plant-based antigenic determinant of H5 HA1 and showed a poor immune response compared to our VLPs results (Spitsin et al., 2009). Mice were immunised with plant-based full-length and truncated H5 recombinant proteins but showed a low humoral immune response in both proteins (Mortimer et al., 2012).

The chicken animal model is our target species and ideal for testing avian influenza vaccines as it is the natural model for the disease (Hemmink et al., 2018, Nguyen et al., 2021). Assessing avian T-cell immune response and detection of immunodominant epitopes should be considered. However, In contrast to mice, assays for measuring immune responses are less readily available in chickens. Tools for assessing avian T-cell immune response and cytokine staining means should be urgently addressed for protection against highly pathogenic viruses, especially AIVs (Dai et al., 2019). Chickens have been previously used to assess the humoral immune response against

plant-based recombinant proteins and vaccines against AIVs (Mortimer et al., 2012, Phan et al., 2020, Smith et al., 2020, Van et al., 2020).

Immunogenicity testing of the pEAQ-HT-H5-VLP using immunised chicken sera showed high specific anti-H5 antibodies with a very low signal in the negative controls and using the pre-immunisation sera. Our results agree with Phan et al. (2020), who collected sera from immunised chickens with plant-based H5 trimers and oligomers and found anti-H5 antibodies in the test group only, while H5 oligomers showed lower binding antibody response than our H5 VLPs using ELISA. In another study, chickens were immunised with two doses of plant-based H6VLPs and gave a low antibody response compared to our results (Smith et al., 2020). The previous studies confirm that VLPs can induce higher levels of binding antibodies than the recombinant proteins, and a booster immunisation dose is important for B cell maturation and increasing antibody longevity.

Frequently, antibodies in AIV plant recombinant protein and VLP trials have been measured using the HI test. The HI test was not performed because HI requires Specified Animal Pathogens Order (SAPO4) containment for using HPAI H5 viruses.

Oligomers of H5 HA showed stronger binding antibodies raised in mice than the H5 trimers (Phan et al., 2018, Phan et al., 2020). Binding antibodies in mice immunised with H6 VLPs showed an HI titre twice that of the inactivated vaccine (Smith et al., 2020). Codon-optimised artificial trimeric H5 protein

showed higher binding antibodies in mice than native H5 protein in HI (Thi Pham et al., 2019). Recombinant H5 proteins showed lower HI binding antibodies in chickens than the recombinant H5 vaccine protein (Ceballo et al., 2017). A high dose of recombinant H5 proteins (15 µg) elicited a higher binding antibody response in mice than 5 µg of recombinant H5 (Major et al., 2015). Full-length H5 recombinant protein elicited higher HI binding antibodies in chickens than the truncated H5 proteins (Mortimer et al., 2012), and H7 full-length protein showed a similar response against rabbit post-vaccination sera with H7 virus using HI (Kanagarajan et al., 2012). The truncated HA1 could not elicit binding antibodies in mice for unknown reasons (Spitsin et al., 2009).

PVNT can measure the neutralising immune response elicited after vaccination against homologous or heterologous strains. PVNT is a safe alternative and more accurate assay to measure the neutralising antibodies without the expensive containment facilities for dealing with the HPAI H5 virus. This is the first time a PVNT assay was used to measure neutralising antibody response against plant-based vaccines. Our results showed an excellent neutralising response against the heterologous strain elicited in mice after three doses and a measurable response after single-dose immunisation in chickens.

PVNT is a very specific test for detecting the neutralisation response in the sera after vaccination, and its results have been correlated with the

haemagglutination test and single radial haemolysis (Huang et al., 2020, Kinsley et al., 2020, Carnell et al., 2021). (Tsai et al., 2009, Delgadillo-Gutiérrez et al., 2022) studied the neutralisation activity of the sera and found that this test is very specific in measuring the neutralisation activity of the sera because the pseudotypes mimic the cell entry and release of the wild-type virus. Del Rosario et al. (2021) produced an HA pseudotype library to test the immunity elicited after vaccination and possible cross-reactivity for universal vaccine production. Moin et al. (2022) immunised mice with HA stem from two influenza groups and found a broadly neutralising antibody and cross-reaction using a PVNT.

The previous studies revealed a high cellular immune response, and the humoral immune response is significant in protecting against infection. Our VLPs showed a high cellular and humoral immune response after three doses in mice and high binding antibodies in chickens after a single dose.

5.1. General discussion

This study used His-tagged HA recombinant proteins and VLPs to produce an efficient vaccine against AIV applicable for use in Egypt. The VLP vaccines were demonstrated to be immunogenic in mice and chickens, generating antibodies detected by ELISA and pseudotyped virus neutralisation test in both species and T-cell responses detected by IFN-gamma ELISpot in the mouse. The first objective was developing a transient plant-based *Agrobacterium*-mediated system for generating VLPs and recombinant proteins using the pEAQ-HT plant expression vector. The following factors were evaluated to increase the protein yield: 1) the codon optimization approach in plants of H9 and H5 and comparison with the non-codon optimised H2, 2) comparison between the efficiency of different the *A. tumefaciens* strains (GV3101 and LBA4404).

5.2. Expression of recombinant proteins and VLPs

In this study, codon optimisation resulted in a higher yield for pEAQ-HT-H9-His-tag and pEAQ-HT-H5-His-tag recombinant proteins than the pEAQ-HT-H2-His-tag proteins, which agrees with (Gustafsson et al., 2004, Webster et al., 2017). The low yield of pEAQ-HT-H2-His-tag recombinant proteins may be attributed to the lack of codon optimisation of the gene. More studies on the pEAQ-HT-H2-His-tag recombinant protein produced in plants and detect the expression level with the specific anti-H2 antibody for more accurate

results about the effect of codon optimisation on the expression levels. More studies are needed to discuss the effect of codon optimisation on protein expression using the same construct.

Agrobacterium GV3101 showed higher transformation efficiency recorded using OD_{600nm} and expression levels than *Agrobacterium* LBA14404, which agrees with previous reports (Liu et al., 2020). These results may be attributed to the different strains of *Agrobacterium* and types of plants that respond differently to different types of target genes (Kraus, 2010, Nyaboga et al., 2014).

The recovery of the VLPs depends mainly on the extraction buffer. In this study, the protein extraction buffer, which contains high salts, failed to recover the VLPs. Then, we modified the protein extraction buffer to contain fewer salts to recover the VLPs, and it gave us excellent results. PBS buffer did not work to recover proteins or VLPs but worked in other studies (Landry et al., 2010).

(IMAC) is a cost-effective method for His-tagged protein purification (Kuo and Chase, 2011, Zhao et al., 2019). In this study, the 6x His-tag was added at the C-terminus of the recombinant proteins, but we struggled to get a good protein amount in the elution because the His-tag was partially exposed. In the case of the pEAQ-HT-H2-His-tag His-tag protein, an *XmaI* restriction site was added between our gene and the His-tag, and we found a good amount

of purified protein and enhancement of the His-tag accessibility, although the expression of pEAQ-HT-H2-His-tag was low.

5.3. Animal trials

For immunogenicity testing of the VLP, partially-purified pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP were emulsified with TiterMax Gold adjuvant and used to vaccinate five weeks old mice with three immunisations. Evaluation of HA-specific antibodies using ELISA revealed an excellent immune response. Three immunisations were enough to elicit an excellent humoral immune response that neutralised the homologous H9 and H5 pseudotypes. After three immunisations, the cellular immune response was detected via ELISpot, revealing an excellent cellular response.

Partially-purified pEAQ-HT-H5-VLPs were emulsified with Montanide ISA 71 VG adjuvant and used to vaccinate SPF chickens at seven days old age, with single immunisation Chicks were bled every week up to six weeks post-immunisation. Evaluation of HA-specific antibodies using ELISA revealed an excellent immune response. Single immunisation elicited an excellent humoral immune response that neutralised around half of the homologous H5 pseudotypes.

Adjuvants are substances added to vaccines to boost the immune response and make it longer lasting (Pulendran et al., 2021). The adjuvants can be

divided into two groups according to the mode of action. The immunostimulant group works by enhancing cytokine secretion by stimulating APCs, such as TLR agonists and saponin. The delivery agent group ensures antigen presentation to the APCs and extends the antigen release for prolonged immune response, known as a depot effect such as mineral salts and emulsions (Burakova et al., 2018). Montanide™ ISA 71 VG from SEPPIC Co. (France) is an oil in water (O/W) emulsion modified from Freund's adjuvant (FA) to induce humoral and cellular immune responses (Klimka et al., 2015). This adjuvant is approved by the Committee for Veterinary Medical Products (CVMP) because it contains no microbial or animal ingredients and is safe for use in vaccines (SEPPIC). Robust cellular and humoral immune responses were induced by H5 VLPs using Montanide™ ISA 71 VG.

TiterMax™Gold is also an improved O/W adjuvant and less toxic than FA. TiterMax™Gold is safe because it does not contain mineral oil or bacterial products (TiterMax®). This adjuvant is designed to maximise antibody responses for monoclonal antibody production and enhance cellular response in clinical animal trials (Fan et al., 2022).

5.4. Study limitations

A drawback of this study is that the lowest effective dose for each VLP vaccine was not determined. Kilany et al. (2016) found that inactivated H9N2 vaccinations with at least 250 HAU per dose induced protective antibody

titres and decreased viral shedding in chickens. In this study, the dose of the pEAQ-HT-H5-VLP was four-fold higher than the inactivated vaccine dose, while the dose for mice was around two-fold higher than the inactivated vaccine dose. The least protective VLP vaccine dose should be evaluated in chickens, and the dose should be evaluated against the homologous and heterologous virus strains. The vaccine efficacy should be evaluated by challenging the immunised chickens using the homologous and heterologous virus strains.

pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP induced high levels of humoral and cellular immune responses in the chicken and mice immunisation trials. Co-expressing pEAQ-HT-H9-VLP and pEAQ-HT-H5-VLP in *N. benthamiana* need to be studied in order to assess the possibility of getting bivalent H9 and H5 VLPs and test them in chicken immunisation trials.

5.5. Future work

VLPs can be used as a potent delivery system to present the target glycoprotein on its surface to boost the immune response elicited in the immunised host. A unique and effective VLP delivery system as a vaccine backbone will save the time and cost needed for vaccine development against emerging diseases. There are many trials to use the HBcAg VLPs as a vaccine delivery system, but they have not been licensed yet. Prof. David Rowland, University of Leeds, UK, has patented an HBcAg VLP system with SUMO-tag affimer (VelcroVax VLPs) that allows the

recombinant proteins to be presented on its surface (Kingston et al., 2023). Presentation of the pEAQ-HT-H9-SUMO-tag and pEAQ-HT-H5-SUMO-tag protein on VelcroVax VLPs will be studied to compare the elicited immune response against VelcroVax VLPs with the one elicited against the H9 and H5 VLPs.

Vaccination designed against avian influenza aims to generate an excellent immune response in the host and prevent virus replication and shedding (Swayne and Kapczynski, 2016). Cost-effectiveness, efficiency after a single vaccination, effectiveness despite the presence of maternal antibodies, compliance with DIVA (differentiation between the infected and vaccinated animals), possible mass application, and need to be updated with the local field strains for the best protection are all requirements for an ideal vaccine for avian influenza (Swayne and Kapczynski, 2016). The IDvet Screen® Influenza is quite expensive commercial kit to differentiate between the infected and vaccinated animals based on the presence of anti-nucleoprotein antibodies in the infected host (Slomka et al., 2019, Souvestre et al., 2019, Amal et al., 2020, Phan et al., 2020, Arbi et al., 2022). These kits were frequently used with reported 99% specificity and 93% sensitivity.

Expressing influenza nucleoprotein in plants and using it is a cheap alternative as a DIVA diagnostic, especially in developing countries.

Expressing IAV nucleoprotein in plants to develop a cheaper version of the IDvet kit that could be used as a DIVA diagnostic in developing countries.

A limitation of plant-based vaccine system application in Egypt is that Egypt does not currently have good manufacturing practice (GMP) facilities for producing these vaccines. Developing a standardised portfolio of plant-based products and the knowledge gained through their commercialization may help developing countries establish GMP facilities for future plant-based vaccines (Morris et al., 2016). Additionally, numerous GMP-compliant biopharming has been created globally, and few plant-based plants have received FDA approval or are, through clinical studies, preparing to produce poultry vaccines from plants (Chen and Lai, 2013, Takeyama et al., 2015, Ward et al., 2020). Therefore, there are not expected to be any prohibitive regulatory obstacles in commercialising plant-produced VLP vaccinations for poultry health.

In ovo application against infectious bursal disease and Marek's disease virus, vaccines are a regular practice in the United States with advantages including reduced stress, contamination, labour costs, and earlier immunity (Negash et al., 2004, Williams and Zedek, 2010). (Schädler et al., 2019) recently reported that the cell culture-based laryngotracheitis VLPs in-Ovo administration vaccine resulted in a good humoral response without any side effects on the growth of chickens reported. The mass application of plant-based influenza VLPs as an edible vaccine or in drinking water should be

considered, which will pave the way for an ideal vaccine. For instance, (Peabody et al., 2017) informed that lyophilisation of VLPs produced in *E. coli* elevated the stability without affecting the immunogenicity, which preserves the vaccine from the freezing and thawing effect and eases the transportation. Plant-based VLPs have many advantages for controlling viruses with high mutation rates and need frequent updates like avian influenza. It is recommended to use this valuable platform for effective, secure, and DIVA-compatible vaccinations for poultry health.

The thesis represents effective and novel plant-based VLPs vaccines against AIV. PVNT was the first time to be used in testing the immunogenicity of plant-based vaccines.

References

AAMIR, U., WERNERY, U., ILYUSHINA, N. & WEBSTER, R. 2007. Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. *Virology,* 361**,** 45-55.

ABDELWHAB, E., ARAFA, A.-S., STECH, J., GRUND, C., STECH, O., GRAEBER-GERBERDING, M., BEER, M., HASSAN, M. K., ALY, M. M. & HARDER, T. C. 2012. Diversifying evolution of highly pathogenic H5N1 avian influenza virus in Egypt from 2006 to 2011. *Virus genes,* 45**,** 14-23.

ABDELWHAB, E., HASSAN, M., ABDEL-MONEIM, A., NAGUIB, M., MOSTAFA, A., HUSSEIN, I., ARAFA, A., ERFAN, A., KILANY, W. & AGOUR, M. 2016. Introduction and enzootic of A/H5N1 in Egypt: Virus evolution, pathogenicity and vaccine efficacy ten years on. *Infection, Genetics and Evolution,* 40**,** 80-90.

ABDELWHAB, E. M. & ABDEL-MONEIM, A. S. 2015. Epidemiology, ecology and gene pool of influenza A virus in Egypt: will Egypt be the epicentre of the next influenza pandemic? *Virulence,* 6**,** 6-18.

ABDUL-CADER, M. S., AHMED-HASSAN, H., AMARASINGHE, A., NAGY, E., SHARIF, S. & ABDUL-CAREEM, M. F. 2017. Toll-like receptor (TLR)21 signalling-mediated antiviral response against avian influenza virus infection correlates with macrophage recruitment and nitric oxide production. *Journal of General Virology,* 98**,** 1209-1223.

ABDUL-CADER, M. S., AMARASINGHE, A. & ABDUL-CAREEM, M. F. 2016. Activation of toll-like receptor signaling pathways leading to nitric oxide-mediated antiviral responses. *Archives of Virology,* 161**,** 2075-86.

ACHDOUT, H., ARNON, T. I., MARKEL, G., GONEN-GROSS, T., KATZ, G., LIEBERMAN, N., GAZIT, R., JOSEPH, A., KEDAR, E. & MANDELBOIM, O. 2003. Enhanced recognition of human NK receptors after influenza virus infection. *The Journal of Immunology,* 171**,** 915-923.

ADA, G. L. & JONES, P. D. 1986. The immune response to influenza infection. *Curr Top Microbiol Immunol,* 128**,** 1-54.

ADDGENE. 2017. *Bacterial Transformation* [Online]. Available: https://www.addgene.org/protocols/bacterial-transformation/ [Accessed 27 June, 2020].

ADEL, A., ARAFA, A., HUSSEIN, H. A. & EL-SANOUSI, A. A. 2017. Molecular and antigenic traits on hemagglutinin gene of avian influenza H9N2 viruses: Evidence of a new escape mutant in Egypt adapted in quails. *Research in Veterinary Science,* 112**,** 132-140.

AFIFI, M. A., EL-KADY, M. F., ZOELFAKAR, S. A. & ABDEL-MONEIM, A. S. 2013. Serological surveillance reveals widespread influenza A H7 and H9 subtypes among chicken flocks in Egypt. *Trop Anim Health Prod,* 45**,** 687-90.

AKACHE, B. & MCCLUSKIE, M. J. 2021. The quantification of antigen-specific T cells by IFN-γ ELISpot. *Vaccine Delivery Technology.* Springer.

AKARSU, H., BURMEISTER, W. P., PETOSA, C., PETIT, I., MÜLLER, C. W., RUIGROK, R. W. & BAUDIN, F. 2003. Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). *Embo j,* 22**,** 4646-55.

AKIRA, S. 2003. Toll-like receptor signaling. *J Biol Chem,* 278**,** 38105-8.

ALARCON, J. B., HARTLEY, A. W., HARVEY, N. G. & MIKSZTA, J. A. 2007. Preclinical evaluation of microneedle technology for intradermal delivery of influenza vaccines. *Clin Vaccine Immunol,* 14**,** 375-81.

ALBERINI, I., DEL TORDELLO, E., FASOLO, A., TEMPERTON, N. J., GALLI, G., GENTILE, C., MONTOMOLI, E., HILBERT, A. K., BANZHOFF, A. & DEL GIUDICE, G. 2009. Pseudoparticle neutralization is a reliable assay to measure immunity and cross-reactivity to H5N1 influenza viruses. *Vaccine,* 27**,** 5998-6003.

ALEXANDER, D. 2003. Report on avian influenza in the Eastern Hemisphere during 1997–2002. *Avian Diseases,* 47**,** 792-797.

ALEXANDER, D., ALLAN, W., PARSONS, D. & PARSONS, G. 1978. The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. *Research in veterinary science,* 24**,** 242-247.

ALEXANDER, D. J. 2007. Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. *Avian diseases,* 51**,** 161-166.

ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature,* 413**,** 732-738.

ALLAN, W., TABI, Z., CLEARY, A. & DOHERTY, P. C. 1990. Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting CD4+ T cells. *J Immunol,* 144**,** 3980-6.

ALLWINN, R., PREISER, W., RABENAU, H., BUXBAUM, S., STÜRMER, M. & DOERR, H. 2002. Laboratory diagnosis of influenza–virology or serology? *Medical microbiology and immunology,* 191**,** 157-160.

ALTENBURG, A. F., KREIJTZ, J.H., DE VRIES, R.D., SONG, F., FUX, R., RIMMELZWAAN, G.F., SUTTER, G. AND VOLZ, A. 2014. Modified vaccinia virus ankara (MVA) as production platform for vaccines against influenza and other viral respiratory diseases. *Viruses,* 6**,** 2735-2761.

ALTPETER, F., BAISAKH, N., BEACHY, R., BOCK, R., CAPELL, T., CHRISTOU, P., DANIELL, H., DATTA, K., DATTA, S. & DIX, P. J. 2005. Particle bombardment and the genetic enhancement of crops: myths and realities. *Molecular Breeding,* 15**,** 305-327.

ALTSTEIN, A. D., GITELMAN, A. K., SMIRNOV, Y. A., PISKAREVA, L. M., ZAKHAROVA, L. G., PASHVYKINA, G. V., SHMAROV, M. M., ZHIRNOV, O. P., VARICH, N. P., ILYINSKII, P. O. & SHNEIDER, A. M. 2006. Immunization with influenza A NP-expressing vaccinia virus recombinant protects mice against experimental infection with human and avian influenza viruses. *Arch Virol,* 151**,** 921-31.

AMAL, E.-B., SAÂDI, N., ASMA, F., MONCEF, B. & OUAFAE, F. F. 2020. Characterization and Phylogenetic Analysis of the Hemagglutinin Gene in H9 Influenza Viruses from Chickens in Morocco from 2017 to 2019. *Avian Diseases,* 64**,** 310-314.

AN, S.-H., SON, S.-E., SONG, J.-H., HONG, S.-M., LEE, C.-Y., LEE, N.-H., JEONG, Y.-J., CHOI, J.-G., LEE, Y.-J. & KANG, H.-M. 2022. Selection of an Optimal Recombinant Egyptian H9N2 Avian Influenza Vaccine Strain for Poultry with High Antigenicity and Safety. *Vaccines,* 10**,** 162.

ANDERSON, R. W., BENNINK, J. R., YEWDELL, J. W., MALOY, W. L. & COLIGAN, J. E. 1992. Influenza basic polymerase 2 peptides are recognized by influenza nucleoprotein-specific cytotoxic T lymphocytes. *Mol Immunol,* 29**,** 1089-96.

ANDREW, M. E., COUPAR, B. E., ADA, G. L. & BOYLE, D. B. 1986. Cell-mediated immune responses to influenza virus antigens expressed by vaccinia virus recombinants. *Microb Pathog,* 1**,** 443-52.

ANTIGUA, K. J. C., BAEK, Y. H., CHOI, W.-S., JEONG, J. H., KIM, E.-H., OH, S., YOON, S.-W., KIM, C., KIM, E.-G. & CHOI, S.-Y. 2022. Multiple HA substitutions in highly pathogenic avian influenza H5Nx viruses contributed to the change in the NA subtype preference. *Virulence,* 13**,** 990-1004.

ARAFA, A., NAGUIB, M., LUTTERMANN, C., SELIM, A., KILANY, W., HAGAG, N., SAMY, A., ABDELHALIM, A., HASSAN, M. & ABDELWHAB, E. 2015. Emergence of a novel cluster of influenza A (H5N1) virus clade 2.2. 1.2 with putative human health impact in Egypt, 2014/15. *Eurosurveillance,* 20**,** 21085.

ARAFA, A. S., HAGAG, N., ERFAN, A., MADY, W., EL-HUSSEINY, M., ADEL, A. & NASEF, S. 2012a. Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. *Virus Genes,* 45**,** 283-94.

ARAFA, A. S., HAGAG, N. M., YEHIA, N., ZANATY, A. M., NAGUIB, M. M. & NASEF, S. A. 2012b. Effect of cocirculation of highly pathogenic avian influenza H5N1 subtype with low pathogenic H9N2 subtype on the spread of infections. *Avian Dis,* 56**,** 849-57.

ARBI, M., LARBI, I., NSIRI, J., EL BEHI, I., REJEB, A., MILED, K., GHRAM, A. & HOUIMEL, M. 2022. Inhibition of avian influenza virus H9N2 infection by antiviral hexapeptides that target viral attachment to epithelial cells. *Virus Research,* 313**,** 198745.

ARRESE, M. & PORTELA, A. 1996. Serine 3 is critical for phosphorylation at the N-terminal end of the nucleoprotein of influenza virus A/Victoria/3/75. *J Virol,* 70**,** 3385-91.

ASANUMA, H., SHARP, M., MAECKER, H. T., MAINO, V. C. & ARVIN, A. M. 2000. Frequencies of memory T cells specific for varicella-zoster virus, herpes simplex virus, and cytomegalovirus by intracellular detection of cytokine expression. *The Journal of infectious diseases,* 181**,** 859-866.

ASHA, K. & KUMAR, B. 2019. Emerging influenza D virus threat: what we know so far! *Journal of Clinical Medicine,* 8**,** 192.

AUGUSTINE, J. J. & HRICIK, D. E. 2012. T-cell immune monitoring by the ELISPOT assay for interferon gamma. *Clinica Chimica Acta,* 413**,** 1359-1363.

AWUNI, J. A., BIANCO, A., DOGBEY, O. J., FUSARO, A., YINGAR, D. T., SALVIATO, A., ABABIO, P. T., MILANI, A., BONFANTE, F. & MONNE, I. 2019. Avian influenza H9N2 subtype in Ghana: virus characterization and evidence of co-infection. *Avian Pathology,* 48**,** 470-476.

BANET-NOACH, C., PERK, S., SIMANOV, L., GREBENYUK, N., ROZENBLUT, E., POKAMUNSKI, S., PIRAK, M., TENDLER, Y. & PANSHIN, A. 2007. H9N2 influenza viruses from Israeli poultry: a five-year outbreak. *Avian diseases,* 51**,** 290-296.

BANKS, J., SPEIDEL, E., MOORE, E., PLOWRIGHT, L., PICCIRILLO, A., CAPUA, I., CORDIOLI, P., FIORETTI, A. & ALEXANDER, D. 2001. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. *Archives of virology,* 146**,** 963-973.

BARBOUR, E., SAGHERIAN, V., SAGHERIAN, N., DANKAR, S., JABER, L., USAYRAN, N. & FARRAN, M. 2006. Avian influenza outbreak in poultry in the Lebanon and transmission to neighbouring farmers and swine. *Vet Ital,* 42**,** 77-85.

BARMAN, S., ADHIKARY, L., CHAKRABARTI, A. K., BERNAS, C., KAWAOKA, Y. & NAYAK, D. P. 2004. Role of transmembrane domain and cytoplasmic tail amino acid sequences of influenza a virus neuraminidase in raft association and virus budding. *J Virol,* 78**,** 5258-69.

BARMAN, S., ALI, A., HUI, E. K., ADHIKARY, L. & NAYAK, D. P. 2001. Transport of viral proteins to the apical membranes and interaction of matrix protein with glycoproteins in the assembly of influenza viruses. *Virus Res,* 77**,** 61-9.

BARMAN, S. & NAYAK, D. P. 2000. Analysis of the transmembrane domain of influenza virus neuraminidase, a type II transmembrane glycoprotein, for apical sorting and raft association. *J Virol,* 74**,** 6538-45.

BASAK, S., PRITCHARD, D. G., BHOWN, A. S. & COMPANS, R. W. 1981. Glycosylation sites of influenza viral glycoproteins: characterization of tryptic glycopeptides from the A/USSR(H1N1) hemagglutinin glycoprotein. *J Virol,* 37**,** 549-58.

BASAK, S., TOMANA, M. & COMPANS, R. W. 1985. Sialic acid is incorporated into influenza hemagglutinin glycoproteins in the absence of viral neuraminidase. *Virus Res,* 2**,** 61-8.

BAUDIN, F., PETIT, I., WEISSENHORN, W. & RUIGROK, R. W. 2001. In vitro dissection of the membrane and RNP binding activities of influenza virus M1 protein. *Virology,* 281**,** 102-8.

BAVAGNOLI, L., CUCUZZA, S., CAMPANINI, G., ROVIDA, F., PAOLUCCI, S., BALDANTI, F. & MAGA, G. 2015. The novel influenza A virus protein PA-X and its naturally deleted variant show different enzymatic properties in comparison to the viral endonuclease PA. *Nucleic acids research,* 43**,** 9405-9417.

BEALE, R., WISE, H., STUART, A., RAVENHILL, B. J., DIGARD, P., RANDOW, F. & MICROBE 2014. A LC3-interacting motif in the influenza A virus M2 protein is required to subvert autophagy and maintain virion stability. *Cell host,* 15**,** 239-247.

BEAN, W., KAWAOKA, Y., WOOD, J., PEARSON, J. & WEBSTER, R. 1985. Characterization of virulent and avirulent A/chicken/Pennsylvania/83 influenza A viruses: potential role of defective interfering RNAs in nature. *Journal of Virology,* 54**,** 151-160.

BEERENS, N., HEUTINK, R., BERGERVOET, S. A., HARDERS, F., BOSSERS, A. & KOCH, G. 2017. Multiple reassorted viruses as cause of highly pathogenic avian influenza A (H5N8) virus epidemic, the Netherlands, 2016. *Emerging infectious diseases,* 23**,** 1974.

BELSER, J. A., BLIXT, O., CHEN, L.-M., PAPPAS, C., MAINES, T. R., VAN HOEVEN, N., DONIS, R., BUSCH, J., MCBRIDE, R. & PAULSON, J. C. 2008. Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. *Proceedings of the National Academy of Sciences,* 105**,** 7558-7563.

BEN-YEDIDIA, T. & ARNON, R. 2005. Towards an epitope-based human vaccine for influenza. *Hum Vaccin,* 1**,** 95-101.

BIER, K., YORK, A. & FODOR, E. 2011. Cellular cap-binding proteins associate with influenza virus mRNAs. *Journal of general virology,* 92**,** 1627-1634.

BLAAS, D., PATZELT, E. & KUECHLER, E. 1982. Cap-recognizing protein of influenza virus. *Virology,* 116**,** 339-348.

BLASS, D., PATZELT, E. & KUECHLER, E. 1982. Identification of the cap binding protein of influenza virus. *Nucleic acids research,* 10**,** 4803-4812.

BODY, M. H., ALRARAWAHI, A. H., ALHUBSY, S. S., SARAVANAN, N., RAJMONY, S. & MANSOOR, M. K. 2015. Characterization of low pathogenic avian influenza virus subtype H9N2 isolated from free-living mynah birds (Acridotheres tristis) in the Sultanate of Oman. *Avian Diseases,* 59**,** 329-334.

BOGS, J., VEITS, J., GOHRBANDT, S., HUNDT, J., STECH, O., BREITHAUPT, A., TEIFKE, J. P., METTENLEITER, T. C. & STECH, J. 2010. Highly pathogenic H5N1 influenza viruses carry virulence determinants beyond the polybasic hemagglutinin cleavage site. *PLoS One,* 5**,** e11826.

BOONE, S. A. & GERBA, C. P. 2007. Significance of fomites in the spread of respiratory and enteric viral disease. *Appl. Environ. Microbiol,* 73**,** 1687-1696.

BOOTH, W. T., SCHLACHTER, C. R., POTE, S., USSIN, N., MANK, N. J., KLAPPER, V., OFFERMANN, L. R., TANG, C., HURLBURT, B. K. & CHRUSZCZ, M. 2018. Impact of an N-terminal polyhistidine tag on protein thermal stability. *ACS omega,* 3**,** 760-768.

BÖTTCHER-FRIEBERTSHÄUSER, E., FREUER, C., SIELAFF, F., SCHMIDT, S., EICKMANN, M., UHLENDORFF, J., STEINMETZER, T., KLENK, H. D. & GARTEN, W. 2010. Cleavage of influenza virus

hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors. *J Virol,* 84**,** 5605-14.

BOTTCHER-FRIEBERTSHAUSER, E., GARTEN, W., MATROSOVICH, M. & KLENK, H. D. 2014. The hemagglutinin: a determinant of pathogenicity. *Curr Top Microbiol Immunol,* 385**,** 3-34.

BOTTCHER, E., MATROSOVICH, T., BEYERLE, M., KLENK, H. D., GARTEN, W. & MATROSOVICH, M. 2006. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. *J Virol,* 80**,** 9896-8.

BOULEY, D., KANANGAT, S., WIRE, W. & ROUSE, B. 1995. Characterization of herpes simplex virus type-1 infection and herpetic stromal keratitis development in IFN-gamma knockout mice. *Journal of immunology (Baltimore, Md.: 1950),* 155**,** 3964-3971.

BOUVIER, N. M. & PALESE, P. 2008. The biology of influenza viruses. *Vaccine,* 26**,** D49-D53.

BRAAM, J., ULMANEN, I. & KRUG, R. M. 1983. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. *Cell,* 34**,** 609-618.

BRANKSTON, G., GITTERMAN, L., HIRJI, Z., LEMIEUX, C. & GARDAM, M. 2007. Transmission of influenza A in human beings. *The Lancet infectious diseases,* 7**,** 257-265.

BROWN, I. H. 2010. Summary of avian influenza activity in Europe, Asia, and Africa, 2006–2009. *Avian diseases,* 54**,** 187-193.

BUI, M., MYERS, J. E. & WHITTAKER, G. R. 2002. Nucleo-cytoplasmic localization of influenza virus nucleoprotein depends on cell density and phosphorylation. *Virus Res,* 84**,** 37-44.

BUI, M., WHITTAKER, G. & HELENIUS, A. 1996. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. *Journal of virology,* 70**,** 8391-8401.

BUI, M., WILLS, E. G., HELENIUS, A. & WHITTAKER, G. R. 2000. Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins. *J Virol,* 74**,** 1781-6.

BULLIDO, R., GÓMEZ-PUERTAS, P., ALBO, C. & PORTELA, A. N. 2000. Several protein regions contribute to determine the nuclear and cytoplasmic localization of the influenza A virus nucleoprotein. *Journal of General Virology,* 81**,** 135-142.

BULLOUGH, P. A., HUGHSON, F. M., SKEHEL, J. J. & WILEY, D. C. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature,* 371**,** 37-43.

BURAKOVA, Y., MADERA, R., MCVEY, S., SCHLUP, J. R. & SHI, J. 2018. Adjuvants for Animal Vaccines. *Viral Immunology,* 31**,** 11-22.

BURGUI, I., ARAGON, T., ORTÍN, J. & NIETO, A. 2003. PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. *Journal of General Virology,* 84**,** 3263-3274.

BUTT, K., SMITH, G. J., CHEN, H., ZHANG, L., LEUNG, Y. C., XU, K., LIM, W., WEBSTER, R. G., YUEN, K. & PEIRIS, J. M. 2005a. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *Journal of clinical microbiology,* 43**,** 5760-5767.

BUTT, T. R., EDAVETTAL, S. C., HALL, J. P. & MATTERN, M. R. 2005b. SUMO fusion technology for difficult-to-express proteins. *Protein Expr Purif,* 43**,** 1-9.

CAMERON, K., GREGORY, V., BANKS, J., BROWN, I., ALEXANDER, D., HAY, A. & LIN, Y. 2000. H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. *Virology,* 278**,** 36-41.

CAO, S., LIU, X., YU, M., LI, J., JIA, X., BI, Y., SUN, L., GAO, G. F. & LIU, W. 2012. A nuclear export signal in the matrix protein of influenza A virus is required for efficient virus replication. *Journal of virology,* 86**,** 4883-4891.

CARNELL, G. W., FERRARA, F., GREHAN, K., THOMPSON, C. P. & TEMPERTON, N. J. 2015. Pseudotype-based neutralization assays for influenza: a systematic analysis. *Frontiers in immunology,* 6**,** 161.

CARNELL, G. W., TROMBETTA, C. M., FERRARA, F., MONTOMOLI, E. & TEMPERTON, N. J. 2021. Correlation of Influenza B Haemagglutination Inhibiton, Single-Radial Haemolysis and Pseudotype-Based Microneutralisation Assays for Immunogenicity Testing of Seasonal Vaccines. *Vaccines (Basel),* 9.

CASTELLS-GRAELLS, R. & LOMONOSSOFF, G. P. 2021. Plant-based production can result in covalent cross-linking of proteins. *Plant Biotechnology Journal,* 19**,** 1095.

CATTOLI, G., MILANI, A., TEMPERTON, N., ZECCHIN, B., BURATIN, A., MOLESTI, E., ALY, M. M., ARAFA, A. & CAPUA, I. 2011. Antigenic drift in H5N1 avian influenza virus in poultry is driven by mutations in major antigenic sites of the hemagglutinin molecule analogous to those for human influenza virus. *J Virol,* 85**,** 8718-24.

CATTOLI, G., MONNE, I., FUSARO, A., JOANNIS, T. M., LOMBIN, L. H., ALY, M. M., ARAFA, A. S., STURM-RAMIREZ, K. M., COUACY-HYMANN, E. & AWUNI, J. A. 2009. Highly pathogenic avian influenza virus subtype H5N1 in Africa: a comprehensive phylogenetic analysis and molecular characterization of isolates. *PLoS One,* 4**,** e4842.

CEBALLO, Y., TIEL, K., LÓPEZ, A., CABRERA, G., PÉREZ, M., RAMOS, O., ROSABAL, Y., MONTERO, C., MENASSA, R. & DEPICKER, A. 2017. High accumulation in tobacco seeds of hemagglutinin antigen from avian (H5N1) influenza. *Transgenic research,* 26**,** 775-789.

CHAMBERS, T. M., KAWAOKA, Y. & WEBSTER, R. G. 1988. Protection of chickens from lethal influenza infection by vaccinia-expressed hemagglutinin. *Virology,* 167**,** 414-421.

CHASE, G. P., RAMEIX-WELTI, M. A., ZVIRBLIENE, A., ZVIRBLIS, G., GÖTZ, V., WOLFF, T., NAFFAKH, N. & SCHWEMMLE, M. 2011. Influenza virus ribonucleoprotein complexes gain preferential access to cellular export machinery through chromatin targeting. *PLoS Pathog,* 7**,** e1002187.

CHEN, B. J., LESER, G. P., MORITA, E. & LAMB, R. A. 2007. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. *Journal of virology,* 81**,** 7111-7123.

CHEN, C. & ZHUANG, X. 2008. Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus. *Proceedings of the National Academy of Sciences,* 105**,** 11790-11795.

CHEN, H., SMITH, G., LI, K., WANG, J., FAN, X., RAYNER, J., VIJAYKRISHNA, D., ZHANG, J., ZHANG, L. & GUO, C. 2006. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proceedings of the National Academy of Sciences,* 103**,** 2845-2850.

CHEN, H., SMITH, G., ZHANG, S., QIN, K., WANG, J., LI, K., WEBSTER, R., PEIRIS, J. & GUAN, Y. 2005. H5N1 virus outbreak in migratory waterfowl. *Nature,* 436**,** 191-192.

CHEN, J. & DENG, Y. M. 2009. Influenza virus antigenic variation, host antibody production and new approach to control epidemics. *Virol J,* 6**,** 30.

CHEN, J., LEE, K. H., STEINHAUER, D. A., STEVENS, D. J., SKEHEL, J. J. & WILEY, D. C. 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell,* 95**,** 409-417.

CHEN, Q.-C., LIU, L., YU, T.-Y., TANG, L., YIN, M.-L., ZHU, W.-H., JIANG, X.-Y. & WANG, H.-Y. 2021. High-level expression and purification of melittin in Escherichia coli using SUMO fusion partner. *International Journal of Peptide Research and Therapeutics,* 27**,** 9-15.

CHEN, Q. & LAI, H. 2013. Plant-derived virus-like particles as vaccines. *Human vaccines & immunotherapeutics,* 9**,** 26-49.

CHEN, W., CALVO, P. A., MALIDE, D., GIBBS, J., SCHUBERT, U., BACIK, I., BASTA, S., O'NEILL, R., SCHICKLI, J. & PALESE, P. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nature medicine,* 7**,** 1306-1312.

CHEN, Z. & KRUG, R. M. 2000. Selective nuclear export of viral mRNAs in influenza-virus-infected cells. *Trends in microbiology,* 8**,** 376-383.

CHEN, Z., LI, Y. & KRUG, R. M. 1999. Influenza A virus NS1 protein targetspoly (A)-binding protein II of the cellular 3′-end processing machinery. *The EMBO journal,* 18**,** 2273-2283.

CHONG, K. T., THANGAVEL, R. R. & TANG, X. 2008. Enhanced expression of murine β-defensins (MBD-1,-2,-3, and-4) in upper and lower airway mucosa of influenza virus infected mice. *Virology,* 380**,** 136-143.

CHOPPIN, P. W. & COMPANS, R. W. 1975. Reproduction of paramyxoviruses. *Comprehensive virology.* Springer.

COHEN, B. & RICHMOND, J. 1982. Electron microscopy of hepatitis B core antigen synthesized in E. coli. *Nature,* 296**,** 677-678.

COLMAN, P. M. & WARD, C. 1985. Structure and diversity of influenza virus neuraminidase. *Current topics in microbiology and immunology***,** 177-255.

COMPANS, R. W. 1973. Influenza virus proteins. II. Association with components of the cytoplasm. *Virology,* 51**,** 56-70.

COUCH, R. B., DECKER, W. K., UTAMA, B., ATMAR, R. L., NINO, D., FENG, J. Q., HALPERT, M. M. & AIR, G. M. 2012. Evaluations for in vitro correlates of immunogenicity of inactivated influenza a H5, H7 and H9 vaccines in humans. *PloS one,* 7.

COUGHLAN, L. 2021. Caught in a trap: How pre-clinical studies in laboratory mice exaggerate vaccine responses. *Cell Reports Medicine,* 2**,** 100484.

COX, R., BROKSTAD, K. & OGRA, P. 2004. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated

and live, attenuated influenza vaccines. *Scandinavian journal of immunology,* 59**,** 1-15.

COX, R. J., MADHUN, A. S., HAUGE, S., SJURSEN, H., MAJOR, D., KUHNE, M., HÖSCHLER, K., SAVILLE, M., VOGEL, F. R. & BARCLAY, W. 2009. A phase I clinical trial of a PER. C6® cell grown influenza H7 virus vaccine. *Vaccine,* 27**,** 1889-1897.

CRAWFORD, J., WILKINSON, B., VOSNESENSKY, A., SMITH, G., GARCIA, M., STONE, H. & PERDUE, M. L. 1999. Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. *Vaccine,* 17**,** 2265-2274.

CREVAR, C. J., CARTER, D. M., LEE, K. Y. & ROSS, T. M. 2015. Cocktail of H5N1 COBRA HA vaccines elicit protective antibodies against H5N1 viruses from multiple clades. *Human vaccines & immunotherapeutics,* 11**,** 572-583.

CROS, J. F. & PALESE, P. 2003. Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses. *Virus Res,* 95**,** 3-12.

D'AOUST, M. A., COUTURE, M. M. J., CHARLAND, N., TREPANIER, S., LANDRY, N., ORS, F. & VÉZINA, L. P. 2010. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. *Plant biotechnology journal,* 8**,** 607-619.

D'AOUST, M. A., LAVOIE, P. O., COUTURE, M. M. J., TRÉPANIER, S., GUAY, J. M., DARGIS, M., MONGRAND, S., LANDRY, N., WARD, B. J. & VÉZINA, L. P. 2008. Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. *Plant biotechnology journal,* 6**,** 930-940.

DAI, M., XU, C., CHEN, W. & LIAO, M. 2019. Progress on chicken T cell immunity to viruses. *Cellular and Molecular Life Sciences,* 76**,** 2779-2788.

DAVIDSON, W. R., YODER, H. W., BRUGH, M. & NETTLES, V. F. 1988. Serological monitoring of eastern wild turkeys for antibodies to Mycoplasma spp. and avian influenza viruses. *Journal of Wildlife Diseases,* 24**,** 348-351.

DAVIS, A. R., BOS, T., UEDA, M., NAYAK, D. P., DOWBENKO, D. & COMPANS, R. W. 1983. Immune response to human influenza virus hemagglutinin expressed in Escherichia coli. *Gene,* 21**,** 273-284.

DE JONG, M. D., SIMMONS, C. P., THANH, T. T., HIEN, V. M., SMITH, G. J., CHAU, T. N. B., HOANG, D. M., CHAU, N. V. V., KHANH, T. H. & DONG,

V. C. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nature medicine,* 12**,** 1203-1207.

DE VRIES, R. D., HERFST, S. & RICHARD, M. 2018. Avian Influenza A Virus Pandemic Preparedness and Vaccine Development. *Vaccines,* 6**,** 46.

DEBIERRE-GROCKIEGO, F., SMITH, T. K., DELBECQ, S., DUCOURNAU, C., LANTIER, L., SCHMIDT, J., BRÈS, V., DIMIER-POISSON, I., SCHWARZ, R. T. & CORNILLOT, E. 2019. Babesia divergens glycosylphosphatidylinositols modulate blood coagulation and induce Th2-biased cytokine profiles in antigen presenting cells. *Biochimie,* 167**,** 135-144.

DEL ROSARIO, J. M. M., DA COSTA, K. A. S., ASBACH, B., FERRARA, F., FERRARI, M., WELLS, D. A., MANN, G. S., AMEH, V. O., SABETA, C. T., BANYARD, A. C., KINSLEY, R., SCOTT, S. D., WAGNER, R., HEENEY, J. L., CARNELL, G. W. & TEMPERTON, N. J. 2021. Exploiting Pan Influenza A and Pan Influenza B Pseudotype Libraries for Efficient Vaccine Antigen Selection. *Vaccines (Basel),* 9.

DELGADILLO-GUTIÉRREZ, K., CASTELÁN-VEGA, J. A., JIMÉNEZ-ALBERTO, A., DEL CARMEN FERNÁNDEZ-LIZÁRRAGA, M., APARICIO-OZORES, G., MONTERRUBIO-LÓPEZ, G. P. & RIBAS-APARICIO, R. M. 2022. Characterization and use in neutralization assays of avian influenza codon-optimized H5 and H7 retroviral pseudotypes. *Journal of Virological Methods,* 300**,** 114391.

DIAS, A., BOUVIER, D., CRÉPIN, T., MCCARTHY, A. A., HART, D. J., BAUDIN, F., CUSACK, S. & RUIGROK, R. W. 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature,* 458**,** 914-918.

DONG, G., LUO, J., ZHANG, H., WANG, C., DUAN, M., DELIBERTO, T. J., NOLTE, D. L., JI, G. & HE, H. 2011. Phylogenetic diversity and genotypical complexity of H9N2 influenza A viruses revealed by genomic sequence analysis. *PloS one,* 6**,** e17212.

DU, L., ZHAO, G., ZHANG, X., LIU, Z., YU, H., ZHENG, B.-J., ZHOU, Y. & JIANG, S. 2010. Development of a safe and convenient neutralization assay for rapid screening of influenza HA-specific neutralizing monoclonal antibodies. *Biochemical and biophysical research communications,* 397**,** 580-585.

DWYER, D. E., SMITH, D. W., CATTON, M. G. & BARR, I. G. 2006. Laboratory diagnosis of human seasonal and pandemic influenza virus infection. *Medical Journal of Australia,* 185**,** S48-S53.

EL-SHESHENY, R., KANDEIL, A., BAGATO, O., MAATOUQ, A. M., MOATASIM, Y., RUBRUM, A., SONG, M.-S., WEBBY, R. J., ALI, M. A. &

KAYALI, G. 2014. Molecular characterization of avian influenza H5N1 virus in Egypt and the emergence of a novel endemic subclade. *The Journal of general virology,* 95**,** 1444.

EL-SHESHENY, R., KANDEIL, A., MOSTAFA, A., ALI, M. A. & WEBBY, R. J. 2021. H5 Influenza viruses in Egypt. *Cold Spring Harbor Perspectives in Medicine,* 11**,** a038745.

EL-SHESHENY, R., KAYALI, G., KANDEIL, A., CAI, Z., BARAKAT, A. B., GHANIM, H. & ALI, M. A. 2012. Antigenic diversity and cross-reactivity of avian influenza H5N1 viruses in Egypt between 2006 and 2011. *Journal of general virology,* 93**,** 2564-2574.

EL-SHESHENY, R., MOATASIM, Y., MAHMOUD, S. H., SONG, Y., EL TAWEEL, A., GOMAA, M., KAMEL, M. N., SAYES, M. E., KANDEIL, A. & LAM, T. T. 2023. Highly Pathogenic Avian Influenza A (H5N1) Virus Clade 2.3. 4.4 b in Wild Birds and Live Bird Markets, Egypt. *Pathogens,* 12**,** 36.

EL HOUADFI, M., FELLAHI, S., NASSIK, S., GUÉRIN, J.-L. & DUCATEZ, M. F. 2016. First outbreaks and phylogenetic analyses of avian influenza H9N2 viruses isolated from poultry flocks in Morocco. *Virology journal,* 13**,** 1-7.

EL SAYES, M., KANDEIL, A., MOATASIM, Y., EL TAWEEL, A., RUBRUM, A., KUTKAT, O., KAMEL, M. N., BADRA, R., BARAKAT, A. B. & MCKENZIE, P. P. 2022. Insights into Genetic Characteristics and Virological Features of Endemic Avian Influenza A (H9N2) Viruses in Egypt from 2017–2021. *Viruses,* 14**,** 1484.

ELBERS, A. R. W., FABRI, T. H. F., DE VRIES, T., DE WIT, J., PIJPERS, A. & KOCH, G. 2004. The highly pathogenic avian influenza A (H7N7) virus epidemic in The Netherlands in 2003—lessons learned from the first five outbreaks. *Avian diseases,* 48**,** 691-705.

ELIZAVETA, B. & ALEXANDRA, G. 2018. Live poultry vaccines against highly pathogenic avian influenza viruses. *Microbiology Independent Research journal,* 5.

ELLIS, J., FLEMING, D. & ZAMBON, M. 1997. Multiplex reverse transcription-PCR for surveillance of influenza A and B viruses in England and Wales in 1995 and 1996. *Journal of clinical microbiology,* 35**,** 2076-2082.

ELLIS, J. S. & ZAMBON, M. C. 2002. Molecular diagnosis of influenza. *Rev Med Virol,* 12**,** 375-89.

ENAMI, M. & ENAMI, K. 1996. Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein. *J Virol,* 70**,** 6653-7.

ENGELHARDT, O. G. & FODOR, E. 2006. Functional association between viral and cellular transcription during influenza virus infection. *Reviews in medical virology,* 16**,** 329-345.

ENGELHARDT, O. G., SMITH, M. & FODOR, E. 2005. Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. *Journal of virology,* 79**,** 5812-5818.

ERFAN, A. M., SELIM, A. A., HELMY, S. A., ERIKSSON, P. & NAGUIB, M. M. 2019. Chicken anaemia virus enhances and prolongs subsequent avian influenza (H9N2) and infectious bronchitis viral infections. *Veterinary microbiology,* 230**,** 123-129.

FAN, J., JIN, S., GILMARTIN, L., TOTH, I., HUSSEIN, W. M. & STEPHENSON, R. J. 2022. Advances in Infectious Disease Vaccine Adjuvants. *Vaccines,* 10**,** 1120.

FARSAD, A. S., MALEKZADEH-SHAFAROUDI, S., MOSHTAGHI, N., FOTOUHI, F. & ZIBAEE, S. 2017. Transient Expression of HA1 Antigen of H5N1 Influenza Virus in Tobacco (Nicotiana tabacum L.) via Agro-infiltration. *Journal of Agricultural Science and Technology,* 19**,** 439-451.

FERRARA, F., DEL ROSARIO, J. M. M., DA COSTA, K. A., KINSLEY, R., SCOTT, S., FEREIDOUNI, S., THOMPSON, C., KELLAM, P., GILBERT, S. & CARNELL, G. 2021. Development of lentiviral vectors pseudotyped with influenza B hemagglutinins: Application in vaccine immunogenicity, mAb potency, and sero-surveillance studies. *Frontiers in Immunology***,** 1904.

FERRARA, F. & TEMPERTON, N. 2018. Pseudotype neutralization assays: from laboratory bench to data analysis. *Methods and protocols,* 1**,** 8.

FIFIS, T., GAMVRELLIS, A., CRIMEEN-IRWIN, B., PIETERSZ, G. A., LI, J., MOTTRAM, P. L., MCKENZIE, I. F. & PLEBANSKI, M. 2004. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J Immunol,* 173**,** 3148-54.

FITCH, W. M., BUSH, R. M., BENDER, C. A. & COX, N. J. 1997. Long term trends in the evolution of H (3) HA1 human influenza type A. *Proceedings of the National Academy of Sciences,* 94**,** 7712-7718.

FODOR, E., DEVENISH, L., ENGELHARDT, O. G., PALESE, P., BROWNLEE, G. G. & GARCÍA-SASTRE, A. 1999. Rescue of influenza A virus from recombinant DNA. *Journal of virology,* 73**,** 9679-9682.

FORMAN, A., PARSONSON, I. & DOUGHTY, W. 1986. The pathogenicity of an avian influenza virus isolated in Victoria. *Australian Veterinary Journal,* 63**,** 294-296.

FORTES, P., BELOSO, A. & ORTIN, J. 1994. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. *The EMBO journal,* 13**,** 704-712.

FRANK, I., PIATAK, M., JR., STOESSEL, H., ROMANI, N., BONNYAY, D., LIFSON, J. D. & POPE, M. 2002. Infectious and whole inactivated simian immunodeficiency viruses interact similarly with primate dendritic cells (DCs): differential intracellular fate of virions in mature and immature DCs. *J Virol,* 76**,** 2936-51.

FUJII, Y., GOTO, H., WATANABE, T., YOSHIDA, T. & KAWAOKA, Y. 2003. Selective incorporation of influenza virus RNA segments into virions. *Proc Natl Acad Sci U S A,* 100**,** 2002-7.

FUSARO, A., MONNE, I., MULATTI, P., ZECCHIN, B., BONFANTI, L., ORMELLI, S., MILANI, A., CECCHETTIN, K., LEMEY, P. & MORENO, A. 2017. Genetic diversity of highly pathogenic avian influenza A (H5N8/H5N5) viruses in Italy, 2016–17. *Emerging Infectious Diseases,* 23**,** 1543.

FUSARO, A., MONNE, I., SALVIATO, A., VALASTRO, V., SCHIVO, A., AMARIN, N. M., GONZALEZ, C., ISMAIL, M. M., AL-ANKARI, A.-R. & AL-BLOWI, M. H. 2011. Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. *Journal of virology,* 85**,** 8413-8421.

GAGNEUX, P., CHERIYAN, M., HURTADO-ZIOLA, N., VAN DER LINDEN, E. C. B., ANDERSON, D., MCCLURE, H., VARKI, A. & VARKI, N. M. 2003. Human-specific regulation of α2–6-linked sialic acids. *Journal of Biological Chemistry,* 278**,** 48245-48250.

GAMBARYAN, A., TUZIKOV, A., BOVIN, N., YAMNIKOVA, S., LVOV, D., WEBSTER, R. & MATROSOVICH, M. 2003. Differences between influenza virus receptors on target cells of duck and chicken and receptor specificity of the 1997 H5N1 chicken and human influenza viruses from Hong Kong. *Avian diseases,* 47**,** 1154-1160.

GAMBARYAN, A., TUZIKOV, A., PISKAREV, V., YAMNIKOVA, S., LVOV, D., ROBERTSON, J., BOVIN, N. & MATROSOVICH, M. 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6′-sialyl (N-acetyllactosamine). *Virology,* 232**,** 345-350.

GAMVRELLIS, A., LEONG, D., HANLEY, J. C., XIANG, S. D., MOTTRAM, P. & PLEBANSKI, M. 2004. Vaccines that facilitate antigen entry into dendritic cells. *Immunol Cell Biol,* 82**,** 506-16.

GARCIA, J.-M. & LAI, J. C. 2011. Production of influenza pseudotyped lentiviral particles and their use in influenza research and diagnosis: an update. *Expert review of anti-infective therapy,* 9**,** 443-455.

GARNER, J. S. A. H. I. C. P. A. C. 1996. Guideline for isolation precautions in hospitals. *Infection Control & Hospital Epidemiology,* 17**,** 54-80.

GARTEN, R. J., DAVIS, C. T., RUSSELL, C. A., SHU, B., LINDSTROM, S., BALISH, A., SESSIONS, W. M., XU, X., SKEPNER, E. & DEYDE, V. 2009. Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. *science,* 325**,** 197-201.

GENBANK. Available: https://www.ncbi.nlm.nih.gov/genbank/ [Accessed 10 May, 2020].

GILES, B. M. & ROSS, T. M. 2011. A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets. *Vaccine,* 29**,** 3043-3054.

GLEBA, Y., KLIMYUK, V. & MARILLONNET, S. 2005. Magnifection—a new platform for expressing recombinant vaccines in plants. *Vaccine,* 23**,** 2042-2048.

GOHRBANDT, S., VEITS, J., HUNDT, J., BOGS, J., BREITHAUPT, A., TEIFKE, J. P., WEBER, S., METTENLEITER, T. C. & STECH, J. 2011. Amino acids adjacent to the haemagglutinin cleavage site are relevant for virulence of avian influenza viruses of subtype H5. *Journal of General Virology,* 92**,** 51-59.

GREENSPAN, D., KRYSTAL, M., NAKADA, S., ARNHEITER, H., LYLES, D. & PALESE, P. 1985. Expression of influenza virus NS2 nonstructural protein in bacteria and localization of NS2 in infected eucaryotic cells. *Journal of virology,* 54**,** 833-843.

GREENSPAN, D., PALESE, P. & KRYSTAL, M. 1988. Two nuclear location signals in the influenza virus NS1 nonstructural protein. *Journal of virology,* 62**,** 3020-3026.

GREGORIADES, A., GUZMAN, G. G. & PAOLETTI, E. 1990. The phosphorylation of the integral membrane (M1) protein of influenza virus. *Virus Res,* 16**,** 27-41.

GRGACIC, E. V. & ANDERSON, D. A. 2006. Virus-like particles: passport to immune recognition. *Methods,* 40**,** 60-5.

GUAN, Y., SHORTRIDGE, K. F., KRAUSS, S. & WEBSTER, R. G. 1999. Molecular characterization of H9N2 influenza viruses: were they the donors of the “internal” genes of H5N1 viruses in Hong Kong? *Proceedings of the National Academy of Sciences,* 96**,** 9363-9367.

GUO, Y., KRAUSS, S., SENNE, D., MO, I., LO, K., XIONG, X., NORWOOD, M., SHORTRIDGE, K., WEBSTER, R. & GUAN, Y. 2000. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology,* 267**,** 279-288.

GUSTAFSSON, C., GOVINDARAJAN, S. & MINSHULL, J. 2004. Codon bias and heterologous protein expression. *Trends in biotechnology,* 22**,** 346-353.

HADDADI, S., THAPA, S., KAMEKA, A. M., HUI, J., CZUB, M., NAGY, E., MUENCH, G. & ABDUL-CAREEM, M. F. 2015. Toll-like receptor 2 ligand, lipoteichoic acid is inhibitory against infectious laryngotracheitis virus infection in vitro and in vivo. *Dev Comp Immunol,* 48**,** 22-32.

HALE, B. G., RANDALL, R. E., ORTÍN, J. & JACKSON, D. 2008. The multifunctional NS1 protein of influenza A viruses. *Journal of general virology,* 89**,** 2359-2376.

HALFORD, W. P., HALFORD, K. J. & PIERCE, A. T. 2005. Mathematical analysis demonstrates that interferons-β and-γ interact in a multiplicative manner to disrupt herpes simplex virus replication. *Journal of theoretical biology,* 234**,** 439-454.

HAMILTON, B. S., WHITTAKER, G. R. & DANIEL, S. 2012. Influenza virus-mediated membrane fusion: determinants of hemagglutinin fusogenic activity and experimental approaches for assessing virus fusion. *Viruses,* 4**,** 1144-1168.

HAMPSON, A. & MACKENZIE, J. 2006. The influenza viruses. *Med J Aust,* 185**,** S39-43.

HARDER, T., MAURER-STROH, S., POHLMANN, A., STARICK, E., HÖRETH-BÖNTGEN, D., ALBRECHT, K., PANNWITZ, G., TEIFKE, J., GUNALAN, V. & LEE, R. T. 2015. Influenza A (H5N8) virus similar to strain in Korea causing highly pathogenic avian influenza in Germany. *Emerging infectious diseases,* 21**,** 860.

HARRISON, S. C. 2015. Viral membrane fusion. *Virology,* 479**,** 498-507.

HATTA, M. & KAWAOKA, Y. 2003. The NB protein of influenza B virus is not necessary for virus replication in vitro. *Journal of virology,* 77**,** 6050-6054.

HELLENS, R., MULLINEAUX, P. & KLEE, H. 2000. Technical focus: a guide to Agrobacterium binary Ti vectors. *Trends in plant science,* 5**,** 446-451.

HEMMINK, J. D., WHITTAKER, C. J. & SHELTON, H. A. 2018. Animal Models in Influenza Research. *In:* YAMAUCHI, Y. (ed.) *Influenza Virus: Methods and Protocols.* New York, NY: Springer New York.

HERFST, S., SCHRAUWEN, E. J., LINSTER, M., CHUTINIMITKUL, S., DE WIT, E., MUNSTER, V. J., SORRELL, E. M., BESTEBROER, T. M., BURKE, D. F. & SMITH, D. J. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *science,* 336**,** 1534-1541.

HILL, N. J., BISHOP, M. A., TROVÃO, N. S., INESON, K. M., SCHAEFER, A. L., PURYEAR, W. B., ZHOU, K., FOSS, A. D., CLARK, D. E. & MACKENZIE, K. G. 2022. Ecological divergence of wild birds drives avian influenza spillover and global spread. *PLoS Pathogens,* 18**,** e1010062.

HOFFMANN, E., STECH, J., GUAN, Y., WEBSTER, R. & PEREZ, D. 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Archives of virology,* 146**,** 2275-2289.

HOMME, P. & EASTERDAY, B. 1970. Avian influenza virus infections. I. Characteristics of influenza A/Turkey/Wisconsin/1966 virus. *Avian diseases***,** 66-74.

HORIMOTO, T., ITO, T., ALEXANDER, D. J. & KAWAOKA, Y. 1995a. Cleavability of hemagglutinin from an extremely virulent strain of avian influenza virus containing a unique cleavage site sequence. *Journal of Veterinary Medical Science,* 57**,** 927-930.

HORIMOTO, T. & KAWAOKA, Y. 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. *Journal of virology,* 68**,** 3120-3128.

HORIMOTO, T. & KAWAOKA, Y. 1997. Biologic effects of introducing additional basic amino acid residues into the hemagglutinin cleavage site of a virulent avian influenza virus. *Virus research,* 50**,** 35-40.

HORIMOTO, T. & KAWAOKA, Y. 2001. Pandemic threat posed by avian influenza A viruses. *Clinical microbiology reviews,* 14**,** 129-149.

HORIMOTO, T. & KAWAOKA, Y. 2005. Influenza: lessons from past pandemics, warnings from current incidents. *Nature Reviews Microbiology,* 3**,** 591.

HORIMOTO, T., NAKAYAMA, K., SMEEKENS, S. P. & KAWAOKA, Y. 1994. Proprotein-processing endoproteases PC6 and furin both activate

hemagglutinin of virulent avian influenza viruses. *Journal of virology,* 68**,** 6074-6078.

HORIMOTO, T., RIVERA, E., PEARSON, J., SENNE, D., KRAUSS, S., KAWAOKA, Y. & WEBSTER, R. 1995b. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. *Virology,* 213**,** 223-230.

HORISBERGER, M. 1980. The large P proteins of influenza A viruses are composed of one acidic and two basic polypeptides. *Virology,* 107**,** 302-305.

HOSSEINI, H., GHALYANCHILANGEROUDI, A., FALLAH MEHRABADI, M. H., SEDIQIAN, M. S., SHAYEGANMEHR, A., GHAFOURI, S. A., MAGHSOUDLOO, H., ABDOLLAHI, H. & FARAHANI, R. K. 2017. Phylogenetic analysis of H9N2 avian influenza viruses in Afghanistan (2016-2017). *Archives of virology,* 162**,** 3161-3165.

HU, Y. C., BENTLEY, W. E., EDWARDS, G. H. & VAKHARIA, V. N. 1999. Chimeric infectious bursal disease virus-like particles expressed in insect cells and purified by immobilized metal affinity chromatography. *Biotechnology and bioengineering,* 63**,** 721-729.

HUANG, R., WANG, A.-R., LIU, Z.-H., LIANG, W., LI, X.-X., TANG, Y.-J., MIAO, Z.-M. & CHAI, T.-J. J. E. J. O. C. M. 2013a. Seroprevalence of avian influenza H9N2 among poultry workers in Shandong Province, China. *European journal of clinical microbiology and infectious diseases,* 32**,** 1347-1351.

HUANG, S., HENDRIKS, W., ALTHAGE, A., HEMMI, S., BLUETHMANN, H., KAMIJO, R., VILČEK, J., ZINKERNAGEL, R. M. & AGUET, M. 1993. Immune response in mice that lack the interferon-γ receptor. *Science,* 259**,** 1742-1745.

HUANG, S. W., TAI, C. H., HSU, Y. M., CHENG, D., HUNG, S. J., CHAI, K. M., WANG, Y. F. & WANG, J. R. 2020. Assessing the application of a pseudovirus system for emerging SARS-CoV-2 and re-emerging avian influenza virus H5 subtypes in vaccine development. *Biomedical Journal,* 43**,** 375-387.

HUANG, X., LIU, T., MULLER, J., LEVANDOWSKI, R. A. & YE, Z. 2001. Effect of influenza virus matrix protein and viral RNA on ribonucleoprotein formation and nuclear export. *Virology,* 287**,** 405-16.

HUANG, Y., LI, Y., BURT, D. W., CHEN, H., ZHANG, Y., QIAN, W., KIM, H., GAN, S., ZHAO, Y. & LI, J. 2013b. The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nature genetics,* 45**,** 776-783.

HUTCHINGS, P. R., CAMBRIDGE, G., TITE, J. P., MEAGER, T. & COOKE, A. 1989. The detection and enumeration of cytokine-secreting cells in mice and man and the clinical application of these assays. *Journal of immunological methods,* 120**,** 1-8.

HUTCHINSON, E. & FODOR, E. 2012. Nuclear import of the influenza A virus transcriptional machinery. *Vaccine,* 30**,** 7353-7358.

HUTCHINSON, E. C., ORR, O. E., LIU, S. M., ENGELHARDT, O. G. & FODOR, E. 2011. Characterization of the interaction between the influenza A virus polymerase subunit PB1 and the host nuclear import factor Ran-binding protein 5. *Journal of general virology,* 92**,** 1859-1869.

HUTCHINSON, E. C., VON KIRCHBACH, J. C., GOG, J. R. & DIGARD, P. 2010. Genome packaging in influenza A virus. *J Gen Virol,* 91**,** 313-28.

ICTV. 2019. *Virus Taxonomy: 2019 Release. [Online]* [Online]. Available: https://talk.ictvonline.org/ictv-reports/ictv_9th_report/negative-sense-rna-viruses-2011/w/negrna_viruses/209/orthomyxoviridae [Accessed 26 May 2020].

IMAI, M., WATANABE, T., HATTA, M., DAS, S. C., OZAWA, M., SHINYA, K., ZHONG, G., HANSON, A., KATSURA, H. & WATANABE, S. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature,* 486**,** 420-428.

INGLIS, S. C., CARROLL, A. R., LAMB, R. A. & MAHY, B. W. 1976. Polypeptides specified by the influenza virus genome: I. Evidence for eight distinct gene products specified by fowl plague virus. *Virology,* 74**,** 489-503.

ITO, T., SUZUKI, Y., SUZUKI, T., TAKADA, A., HORIMOTO, T., WELLS, K., KIDA, H., OTSUKI, K., KISO, M. & ISHIDA, H. 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the α2, 3 linkage is associated with intestinal replication of influenza A virus in ducks. *Journal of virology,* 74**,** 9300-9305.

IWATSUKI-HORIMOTO, K., HORIMOTO, T., FUJII, Y. & KAWAOKA, Y. 2004. Generation of influenza A virus NS2 (NEP) mutants with an altered nuclear export signal sequence. *J Virol,* 78**,** 10149-55.

JAGGER, B., WISE, H., KASH, J., WALTERS, K.-A., WILLS, N., XIAO, Y.-L., DUNFEE, R., SCHWARTZMAN, L., OZINSKY, A. & BELL, G. 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science,* 337**,** 199-204.

JEONG, H. & SEONG, B. L. 2017. Exploiting virus-like particles as innovative vaccines against emerging viral infections. *J Microbiol,* 55**,** 220-230.

JI, N. & FORSTHUBER, T. G. 2016. ELISPOT techniques. *Multiple Sclerosis: Methods and Protocols***,** 63-71.

JIANG, W.-M., WANG, S.-C., LIU, H.-L., YU, J.-M., DU, X., HOU, G.-Y., LI, J.-P., LIU, S., WANG, K.-C. & ZHUANG, Q.-Y. 2014. Evaluation of avian influenza virus isolated from ducks as a potential live vaccine candidate against novel H7N9 viruses. *Vaccine,* 32**,** 6433-6439.

JOHNSON, N. P. & MUELLER, J. 2002. Updating the accounts: global mortality of the 1918-1920" Spanish" influenza pandemic. *Bulletin of the History of Medicine,* 76**,** 105-115.

KAMMON, A., HEIDARI, A., DAYHUM, A., ELDAGHAYES, I., SHARIF, M., MONNE, I., CATTOLI, G., ASHEG, A., FARHAT, M. & KRAIM, E. 2015. Characterization of avian influenza and Newcastle disease viruses from poultry in Libya. *Avian Diseases,* 59**,** 422-430.

KANAGARAJAN, S., TOLF, C., LUNDGREN, A., WALDENSTRÖM, J. & BRODELIUS, P. E. 2012. Transient expression of hemagglutinin antigen from low pathogenic avian influenza A (H7N7) in Nicotiana benthamiana. *PloS one,* 7**,** e33010.

KANDEIL, A., EL-SHESHENY, R., MAATOUQ, A., MOATASIM, Y., CAI, Z., MCKENZIE, P., WEBBY, R., KAYALI, G. & ALI, M. A. 2017a. Novel reassortant H9N2 viruses in pigeons and evidence for antigenic diversity of H9N2 viruses isolated from quails in Egypt. *The Journal of general virology,* 98**,** 548.

KANDEIL, A., EL-SHESHENY, R., MAATOUQ, A. M., MOATASIM, Y., SHEHATA, M. M., BAGATO, O., RUBRUM, A., SHANMUGANATHAM, K., WEBBY, R. J. & ALI, M. A. 2014. Genetic and antigenic evolution of H9N2 avian influenza viruses circulating in Egypt between 2011 and 2013. *Archives of virology,* 159**,** 2861-2876.

KANDEIL, A., HICKS, J. T., YOUNG, S. G., EL TAWEEL, A. N., KAYED, A. S., MOATASIM, Y., KUTKAT, O., BAGATO, O., MCKENZIE, P. P., CAI, Z., BADRA, R., KUTKAT, M., BAHL, J., WEBBY, R. J., KAYALI, G. & ALI, M. A. 2019. Active surveillance and genetic evolution of avian influenza viruses in Egypt, 2016–2018. *Emerging Microbes & Infections,* 8**,** 1370-1382.

KANDEIL, A., KAYED, A., MOATASIM, Y., WEBBY, R. J., MCKENZIE, P. P., KAYALI, G. & ALI, M. A. 2017b. Genetic characterization of highly pathogenic avian influenza A H5N8 viruses isolated from wild birds in Egypt. *The Journal of general virology,* 98**,** 1573.

KARRON, R. A., TALAAT, K., LUKE, C., CALLAHAN, K., THUMAR, B., DILORENZO, S., MCAULIFFE, J., SCHAPPELL, E., SUGUITAN, A. & MILLS, K. 2009. Evaluation of two live attenuated cold-adapted H5N1 influenza virus vaccines in healthy adults. *Vaccine,* 27**,** 4953-4960.

KARUPIAH, G., FREDRICKSON, T. N., HOLMES, K. L., KHAIRALLAH, L. & BULLER, R. 1993. Importance of interferons in recovery from mousepox. *Journal of virology,* 67**,** 4214-4226.

KATZ, J. M., LIM, W., BRIDGES, C. B., ROWE, T., HU-PRIMMER, J., LU, X., ABERNATHY, R. A., CLARKE, M., CONN, L. & KWONG, H. 1999. Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts. *Journal of Infectious Diseases,* 180**,** 1763-1770.

KATZE, M. G., CHEN, Y.-T. & KRUG, R. M. 1984. Nuclear-cytoplasmic transport and VAI RNA-independent translation of influenza viral messenger RNAs in late adenovirus-infected cells. *Cell,* 37**,** 483-490.

KATZE, M. G., DECORATO, D. & KRUG, R. M. 1986a. Cellular mRNA translation is blocked at both initiation and elongation after infection by influenza virus or adenovirus. *Journal of virology,* 60**,** 1027-1039.

KATZE, M. G., DETJEN, B. M., SAFER, B. & KRUG, R. M. 1986b. Translational control by influenza virus: suppression of the kinase that phosphorylates the alpha subunit of initiation factor eIF-2 and selective translation of influenza viral mRNAs. *Molecular and cellular biology,* 6**,** 1741-1750.

KATZE, M. G., HE, Y. & GALE, M. 2002. Viruses and interferon: a fight for supremacy. *Nature Reviews Immunology,* 2**,** 675-687.

KAWAOKA, Y., NAEVE, C. W. & WEBSTER, R. G. 1984. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? *Virology,* 139**,** 303-316.

KAWAOKA, Y. & WEBSTER, R. G. 1988. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. *Proc Natl Acad Sci U S A,* 85**,** 324-8.

KAYALI, G., EL-SHESHENY, R., KUTKAT, M. A., KANDEIL, A. M., MOSTAFA, A., DUCATEZ, M. F., MCKENZIE, P. P., GOVORKOVA, E. A., NASRAA, M. H. & WEBSTER, R. G. 2011a. Continuing threat of influenza (H5N1) virus circulation in Egypt. *Emerging Infectious Diseases,* 17**,** 2306.

KAYALI, G., KANDEIL, A., EL-SHESHENY, R., KAYED, A. S., MAATOUQ, A. M., CAI, Z., MCKENZIE, P. P., WEBBY, R. J., EL REFAEY, S. &

KANDEEL, A. 2016. Avian influenza A (H5N1) virus in Egypt. *Emerging infectious diseases,* 22**,** 379.

KAYALI, G., WEBBY, R. J., DUCATEZ, M. F., EL SHESHENY, R. A., KANDEIL, A. M., GOVORKOVA, E. A., MOSTAFA, A. & ALI, M. A. 2011b. The epidemiological and molecular aspects of influenza H5N1 viruses at the human-animal interface in Egypt. *PloS one,* 6**,** e17730.

KAYED, A., KANDEIL, A., EL SHESHENY, R., ALI, M. & KAYALI, G. 2016. Active surveillance of avian influenza viruses in Egyptian poultry, 2015. *EMHJ-Eastern Mediterranean Health Journal,* 22**,** 553-557.

KHALAJ-HEDAYATI, A., CHUA, C. L. L., SMOOKER, P. & LEE, K. W. 2020. Nanoparticles in influenza subunit vaccine development: Immunogenicity enhancement. *Influenza Other Respir Viruses,* 14**,** 92-101.

KHAN, F. H. 2009. *The elements of immunology*, Pearson Education India.

KHANNA, M., KUMAR, P., CHOUDHARY, K., KUMAR, B. & VIJAYAN, V. 2008. Emerging influenza virus: a global threat. *Journal of biosciences,* 33**,** 475-482.

KIDO, H., OKUMURA, Y., TAKAHASHI, E., PAN, H.-Y., WANG, S., YAO, D., YAO, M., CHIDA, J. & YANO, M. 2012. Role of host cellular proteases in the pathogenesis of influenza and influenza-induced multiple organ failure. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics,* 1824**,** 186-194.

KILANY, W. H., ALI, A., BAZID, A.-H. I., EL-DEEB, A. H., EL-ABIDEEN, M. A. Z., SAYED, M. E. & EL-KADY, M. F. 2016. A dose-response study of inactivated low pathogenic avian influenza H9N2 virus in specific-pathogen-free and commercial broiler chickens. *Avian diseases,* 60**,** 256-261.

KIM, S. H. & SAMAL, S. K. 2019. Innovation in Newcastle Disease Virus Vectored Avian Influenza Vaccines. *Viruses,* 11.

KINGSTON, N. J., GREHAN, K., SNOWDEN, J. S., HASSALL, M., ALZAHRANI, J., PAESEN, G. C., SHERRY, L., HAYWARD, C., ROE, A. & STEPHEN, S. 2023. VelcroVax: a “Bolt-On” Vaccine Platform for Glycoprotein Display. *mSphere***,** e00568-22.

KINSLEY, R., PRONOST, S., DE BOCK, M., TEMPERTON, N., DALY, J. M., PAILLOT, R. & SCOTT, S. 2020. Evaluation of a Pseudotyped Virus Neutralisation Test for the Measurement of Equine Influenza Virus-Neutralising Antibody Responses Induced by Vaccination and Infection. *Vaccines (Basel),* 8.

KLENK, H.-D., ROTT, R., ORLICH, M. & BLÖDORN, J. 1975. Activation of influenza A viruses by trypsin treatment. *Virology,* 68**,** 426-439.

KLENK, H. D., WAGNER, R., HEUER, D. & WOLFF, T. 2002. Importance of hemagglutinin glycosylation for the biological functions of influenza virus. *Virus Res,* 82**,** 73-5.

KLENK, H. D., WÖLLERT, W., ROTT, R. & SCHOLTISSEK, C. 1974. Association of influenza virus proteins with cytoplasmic fractions. *Virology,* 57**,** 28-41.

KLEYHEEG, E., SLATERUS, R., BODEWES, R., RIJKS, J. M., SPIERENBURG, M. A., BEERENS, N., KELDER, L., POEN, M. J., STEGEMAN, J. A. & FOUCHIER, R. A. 2017. Deaths among wild birds during highly pathogenic avian influenza A (H5N8) virus outbreak, the Netherlands. *Emerging infectious diseases,* 23**,** 2050.

KLIMKA, A., MICHELS, L., GLOWALLA, E., TOSETTI, B., KRÖNKE, M. & KRUT, O. 2015. Montanide ISA 71 VG is Advantageous to Freund's Adjuvant in Immunization Against S. aureus Infection of Mice. *Scand J Immunol,* 81**,** 291-7.

KOLETZKI, D., BIEL, S. S., MEISEL, H., NUGEL, E., GELDERBLOM, H. R., KRÜGER, D. H. & ULRICH, R. 1999. HBV core particles allow the insertion and surface exposure of the entire potentially protective region of Puumala hantavirus nucleocapsid protein. *Biol Chem,* 380**,** 325-33.

KOOPMANS, M., WILBRINK, B., CONYN, M., NATROP, G., VAN DER NAT, H., VENNEMA, H., MEIJER, A., VAN STEENBERGEN, J., FOUCHIER, R. & OSTERHAUS, A. 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *The Lancet,* 363**,** 587-593.

KORNFELD, R. & KORNFELD, S. 1985. Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem,* 54**,** 631-64.

KOZAK, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic acids research,* 15**,** 8125-8148.

KOZAK, M. 1989. The scanning model for translation: an update. *The Journal of cell biology,* 108**,** 229-241.

KRAIDI, Q. A., MADADGAR, O., GHALYANCHI LANGEROUDI, A. & KARIMI, V. 2017. Genetic analysis of H9N2 avian influenza viruses circulated in broiler flocks: a case study in Iraq in 2014–2015. *Virus genes,* 53**,** 205-214.

KRAMMER, F. & PALESE, P. 2015. Advances in the development of influenza virus vaccines. *Nature reviews Drug discovery,* 14**,** 167-182.

KRAUS, J. 2010. Concepts of marker genes for plants. *Genetic Modification of Plants: Agriculture, Horticulture and Forestry***,** 39-60.

KREIJTZ, J., BODEWES, R., VAN AMERONGEN, G., KUIKEN, T., FOUCHIER, R., OSTERHAUS, A. & RIMMELZWAAN, G. 2007. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. *Vaccine,* 25**,** 612-620.

KREIJTZ, J., FOUCHIER, R. & RIMMELZWAAN, G. 2011. Immune responses to influenza virus infection. *Virus research,* 162**,** 19-30.

KREIJTZ, J. H., GOEIJENBIER, M., MOESKER, F. M., VAN DEN DRIES, L., GOEIJENBIER, S., DE GRUYTER, H. L., ... & FOUCHIER, R. A. 2014. Safety and immunogenicity of a modified-vaccinia-virus-Ankara-based influenza A H5N1 vaccine: a randomised, double-blind phase 1/2a clinical trial. *The Lancet Infectious Diseases,* 14**,** 1196-1207.

KREIJTZ, J. H., SUEZER, Y., DE MUTSERT, G., VAN AMERONGEN, G., SCHWANTES, A., VAN DEN BRAND, J.M., FOUCHIER, R.A., LÖWER, J., OSTERHAUS, A.D., SUTTER, G. AND RIMMELZWAAN, G.F., 2009. , 4(11). 2009a. MVA-based H5N1 vaccine affords cross-clade protection in mice against influenza A/H5N1 viruses at low doses and after single immunization. *PloS one,* 4.

KREIJTZ, J. H. C. M., SUEZER, Y., DE MUTSERT, G., VAN DEN BRAND, J. M. A., VAN AMERONGEN, G., SCHNIERLE, B. S., ... & SUTTER, G. 2009b. Recombinant modified vaccinia virus Ankara expressing the hemagglutinin gene confers protection against homologous and heterologous H5N1 influenza virus infections in macaques. *The Journal of infectious diseases,* 199**,** 405-413.

KUCHIPUDI, S. V., NELLI, R., WHITE, G. A., BAIN, M., CHANG, K. C. & DUNHAM, S. 2009. Differences in influenza virus receptors in chickens and ducks: implications for interspecies transmission. *Journal of molecular and genetic medicine: an international journal of biomedical research,* 3**,** 143.

KUNDU, A., AVALOS, R. T., SANDERSON, C. M. & NAYAK, D. P. 1996. Transmembrane domain of influenza virus neuraminidase, a type II protein, possesses an apical sorting signal in polarized MDCK cells. *J Virol,* 70**,** 6508-15.

KUO, W.-H. K. & CHASE, H. A. 2011. Exploiting the interactions between poly-histidine fusion tags and immobilized metal ions. *Biotechnology letters,* 33**,** 1075-1084.

KUSHNIR, N., STREATFIELD, S. J. & YUSIBOV, V. 2012. Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine,* 31**,** 58-83.

KWON, K.-C., CHAN, H.-T., LEÓN, I. R., WILLIAMS-CARRIER, R., BARKAN, A. & DANIELL, H. 2016. Codon optimization to enhance expression yields insights into chloroplast translation. *Plant physiology,* 172**,** 62-77.

LADDY, D. J., YAN, J., CORBITT, N., KOBASA, D., KOBINGER, G. P. & WEINER, D. B. 2007. Immunogenicity of novel consensus-based DNA vaccines against avian influenza. *Vaccine,* 25**,** 2984-2989.

LADDY, D. J., YAN, J., KHAN, A. S., ANDERSEN, H., COHN, A., GREENHOUSE, J., LEWIS, M., MANISCHEWITZ, J., KING, L. R. & GOLDING, H. 2009. Electroporation of synthetic DNA antigens offers protection in nonhuman primates challenged with highly pathogenic avian influenza virus. *Journal of virology,* 83**,** 4624-4630.

LADDY, D. J., YAN, J., KUTZLER, M., KOBASA, D., KOBINGER, G. P., KHAN, A. S., GREENHOUSE, J., SARDESAI, N. Y., DRAGHIA-AKLI, R. & WEINER, D. B. 2008. Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens. *PloS one,* 3.

LAKADAMYALI, M., RUST, M. J., BABCOCK, H. P. & ZHUANG, X. 2003. Visualizing infection of individual influenza viruses. *Proceedings of the National Academy of Sciences,* 100**,** 9280-9285.

LAMB, R. 1974. *Aspects of the Structure and Replication of Sendai Virus.* University of Cambridge.

LAMB, R. A. & CHOPPIN, P. W. 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides, from RNA produced by primary transcription. *Virology,* 74**,** 504-519.

LANDRY, N., WARD, B. J., TRÉPANIER, S., MONTOMOLI, E., DARGIS, M., LAPINI, G. & VÉZINA, L.-P. 2010. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PloS one,* 5.

LAZAROWITZ, S. G. & CHOPPIN, P. W. 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. *Virology,* 68**,** 440-454.

LEE, C.-W., SENNE, D. A. & SUAREZ, D. L. 2004. Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (Differentiating Infected from Vaccinated Animals) strategy for the control of avian influenza. *Vaccine,* 22**,** 3175-3181.

LEE, D.-H., BERTRAN, K., KWON, J.-H. & SWAYNE, D. E. 2017. Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3. 4.4. *Journal of veterinary science,* 18**,** 269-280.

LEE, D.-H., TORCHETTI, M. K., WINKER, K., IP, H. S., SONG, C.-S. & SWAYNE, D. E. 2015. Intercontinental spread of Asian-origin H5N8 to North America through Beringia by migratory birds. *Journal of virology,* 89**,** 6521-6524.

LEE, K. W., TEY, B. T., HO, K. L. & TAN, W. S. 2012. Delivery of chimeric hepatitis B core particles into liver cells. *J Appl Microbiol,* 112**,** 119-31.

LEE, N., WONG, C., CHAN, P., LUN, S., LUI, G., WONG, B., HUI, D., LAM, C., COCKRAM, C. & CHOI, K. 2007. Hypercytokinemia and hyperactivation of phospho-p38 mitogen-activated protein kinase in severe human influenza A virus infection. *Clinical infectious diseases,* 45**,** 723-731.

LEE, S., GIERYNSKA, M., EO, S. K., KUKLIN, N. & ROUSE, B. T. 2003. Influence of DNA encoding cytokines on systemic and mucosal immunity following genetic vaccination against herpes simplex virus. *Microbes and infection,* 5**,** 571-578.

LESER, G. P. & LAMB, R. A. 2005. Influenza virus assembly and budding in raft-derived microdomains: a quantitative analysis of the surface distribution of HA, NA and M2 proteins. *Virology,* 342**,** 215-27.

LEUZINGER, K., DENT, M., HURTADO, J., STAHNKE, J., LAI, H., ZHOU, X. & CHEN, Q. 2013. Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. *JoVE***,** e50521.

LEVINE, M. Z., HOLIDAY, C., LIU, F., JEFFERSON, S., GILLIS, E., BELLAMY, A. R., TUMPEY, T. & KATZ, J. M. 2017. Cross-reactive antibody responses to novel H5Nx influenza viruses following homologous and heterologous prime-boost vaccination with a prepandemic stockpiled A (H5N1) vaccine in humans. *The Journal of infectious diseases,* 216**,** S555-S559.

LEYSON, C. M., YOUK, S., FERREIRA, H. L., SUAREZ, D. L. & PANTIN-JACKWOOD, M. 2021. Multiple gene segments are associated with enhanced virulence of clade 2.3. 4.4 H5N8 highly pathogenic avian influenza virus in mallards. *Journal of virology,* 95**,** e00955-21.

LI, C., BU, Z. & CHEN, H. 2014. Avian influenza vaccines against H5N1 'bird flu'. *Trends in biotechnology,* 32**,** 147-156.

LI, C., WANG, S., BING, G., CARTER, R. A., WANG, Z., WANG, J., WANG, C., WANG, L., WU, G. & WEBSTER, R. G. 2017. Genetic evolution of influenza H9N2 viruses isolated from various hosts in China from 1994 to 2013. *Emerging microbes & infections,* 6**,** 1-11.

LI, Y.-T., SU, Y. C. & SMITH, G. J. 2021. H5Nx Viruses Emerged during the Suppression of H5N1 Virus Populations in Poultry. *Microbiology spectrum,* 9**,** e01309-21.

LIN, S., NAIM, H. Y., RODRIGUEZ, A. C. & ROTH, M. G. 1998. Mutations in the middle of the transmembrane domain reverse the polarity of transport of the influenza virus hemagglutinin in MDCK epithelial cells. *J Cell Biol,* 142**,** 51-7.

LIN, Y., SHAW, M., GREGORY, V., CAMERON, K., LIM, W., KLIMOV, A., SUBBARAO, K., GUAN, Y., KRAUSS, S. & SHORTRIDGE, K. 2000. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. *Proceedings of the National Academy of Sciences,* 97**,** 9654-9658.

LINDSAY, B. J., BONAR, M. M., COSTAS-CANCELAS, I. N., HUNT, K., MAKARKOV, A. I., CHIERZI, S., KRAWCZYK, C. M., LANDRY, N., WARD, B. J. & ROUILLER, I. 2018. Morphological characterization of a plant-made virus-like particle vaccine bearing influenza virus hemagglutinins by electron microscopy. *Vaccine,* 36**,** 2147-2154.

LINSTER, M., VAN BOHEEMEN, S., DE GRAAF, M., SCHRAUWEN, E. J., LEXMOND, P., MÄNZ, B., BESTEBROER, T. M., BAUMANN, J., VAN RIEL, D. & RIMMELZWAAN, G. F. 2014. Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus. *Cell,* 157**,** 329-339.

LIU, J., XIAO, H., LEI, F., ZHU, Q., QIN, K., ZHANG, X.-W., ZHANG, X.-L., ZHAO, D., WANG, G. & FENG, Y. 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science,* 309**,** 1206-1206.

LIU, M., WOOD, J. M., ELLIS, T., KRAUSS, S., SEILER, P., JOHNSON, C., HOFFMANN, E., HUMBERD, J., HULSE, D. & ZHANG, Y. 2003. Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. *Virology,* 314**,** 580-590.

LIU, S., MA, J., LIU, H., GUO, Y., LI, W. & NIU, S. 2020. An efficient system for Agrobacterium-mediated transient transformation in Pinus tabuliformis. *Plant Methods,* 16**,** 1-9.

LOH, H.-S. 2014. Optimizations of Laboratory-scale Vacuum-assisted Agroinfiltration for Delivery of a Transgene in Nicotiana benthamiana.

LONG, J. S., GIOTIS, E. S., MONCORGÉ, O., FRISE, R., MISTRY, B., JAMES, J., MORISSON, M., IQBAL, M., VIGNAL, A. & SKINNER, M. A. 2016. Species difference in ANP32A underlies influenza A virus polymerase host restriction. *Nature,* 529**,** 101-104.

LU, Y., QIAN, X.-Y. & KRUG, R. M. 1994. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. *Genes & Development,* 8**,** 1817-1828.

LUA, L. H., CONNORS, N. K., SAINSBURY, F., CHUAN, Y. P., WIBOWO, N. & MIDDELBERG, A. P. 2014. Bioengineering virus-like particles as vaccines. *Biotechnol Bioeng,* 111**,** 425-40.

LUCZO, J. M., STAMBAS, J., DURR, P. A., MICHALSKI, W. P. & BINGHAM, J. 2015. Molecular pathogenesis of H5 highly pathogenic avian influenza: the role of the haemagglutinin cleavage site motif. *Reviews in medical virology,* 25**,** 406-430.

LUND, J. M., ALEXOPOULOU, L., SATO, A., KAROW, M., ADAMS, N. C., GALE, N. W., IWASAKI, A. & FLAVELL, R. A. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proceedings of the National Academy of Sciences,* 101**,** 5598-5603.

LUPIANI, B. & REDDY, S. M. 2009. The history of avian influenza. *Comparative Immunology, Microbiology and Infectious Diseases,* 32**,** 311-323.

LYCETT, S. J., POHLMANN, A., STAUBACH, C., CALIENDO, V., WOOLHOUSE, M., BEER, M., KUIKEN, T., VAN BORM, S., BREED, A. & BRIAND, F.-X. 2020. Genesis and spread of multiple reassortants during the 2016/2017 H5 avian influenza epidemic in Eurasia. *Proceedings of the National Academy of Sciences,* 117**,** 20814-20825.

MA, H. & CHEN, G. 2005. Gene transfer technique. *Nature and Science,* 3**,** 25-31.

MACKENZIE, J. S. & JEGGO, M. 2019. The One Health approach—Why is it so important? *Tropical medicine and infectious disease,* 4**,** 88.

MACMICKING, J., XIE, Q. W. & NATHAN, C. 1997. Nitric oxide and macrophage function. *Annu Rev Immunol,* 15**,** 323-50.

MAIER, H. J., KASHIWAGI, T., HARA, K. & BROWNLEE, G. G. 2008. Differential role of the influenza A virus polymerase PA subunit for vRNA and cRNA promoter binding. *Virology,* 370**,** 194-204.

MAJOR, D., CHICHESTER, J. A., PATHIRANA, R. D., GUILFOYLE, K., SHOJI, Y., GUZMAN, C. A., YUSIBOV, V. & COX, R. J. 2015. Intranasal vaccination with a plant-derived H5 HA vaccine protects mice and ferrets against highly pathogenic avian influenza virus challenge. *Human vaccines & immunotherapeutics,* 11**,** 1235-1243.

MANUEL-CABRERA, C. A., VALLEJO-CARDONA, A. A., PADILLA-CAMBEROS, E., HERNÁNDEZ-GUTIÉRREZ, R., HERRERA-RODRÍGUEZ, S. E. & GUTIÉRREZ-ORTEGA, A. 2016. Self-assembly of hexahistidine-tagged tobacco etch virus capsid protein into microfilaments that induce IgG2-specific response against a soluble porcine reproductive and respiratory syndrome virus chimeric protein. *Virology Journal,* 13**,** 1-6.

MARBLESTONE, J. G., EDAVETTAL, S. C., LIM, Y., LIM, P., ZUO, X. & BUTT, T. R. 2006. Comparison of SUMO fusion technology with traditional gene fusion systems: enhanced expression and solubility with SUMO. *Protein Sci,* 15**,** 182-9.

MARGINE, I. & KRAMMER, F. 2014. Animal models for influenza viruses: implications for universal vaccine development. *Pathogens,* 3**,** 845-874.

MARTIN, K. & HELENIUS, A. 1991a. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell,* 67**,** 117-30.

MARTIN, K. & HELENIUS, A. 1991b. Transport of incoming influenza virus nucleocapsids into the nucleus. *Journal of virology,* 65**,** 232-244.

MARTIN, K. & HELENIUST, A. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell,* 67**,** 117-130.

MATROSOVICH, M., GAMBARYAN, A., TENEBERG, S., PISKAREV, V., YAMNIKOVA, S., LVOV, D., ROBERTSON, J. & KARLSSON, K.-A. 1997. Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology,* 233**,** 224-234.

MATROSOVICH, M., TUZIKOV, A., BOVIN, N., GAMBARYAN, A., KLIMOV, A., CASTRUCCI, M. R., DONATELLI, I. & KAWAOKA, Y. 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *Journal of virology,* 74**,** 8502-8512.

MATROSOVICH, M. N., MATROSOVICH, T. Y., GRAY, T., ROBERTS, N. A. & KLENK, H.-D. 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proceedings of the National Academy of Sciences,* 101**,** 4620-4624.

MATSUOKA, Y., MATSUMAE, H., KATOH, M., EISFELD, A. J., NEUMANN, G., HASE, T., GHOSH, S., SHOEMAKER, J. E., LOPES, T. J. S., WATANABE, T., WATANABE, S., FUKUYAMA, S., KITANO, H. & KAWAOKA, Y. 2013. A comprehensive map of the influenza A virus replication cycle. *BMC Systems Biology,* 7**,** 97.

MAUDSLEY, D. J. & MORRIS, A. G. 1989. Regulation of IFN-gamma-induced host cell MHC antigen expression by Kirsten MSV and MLV. II. Effects on class II antigen expression. *Immunology,* 67**,** 26-31.

MAURER-STROH, S., LEE, R. T., GUNALAN, V. & EISENHABER, F. 2013. The highly pathogenic H7N3 avian influenza strain from July 2012 in Mexico acquired an extended cleavage site through recombination with host 28S rRNA. *Virology journal,* 10**,** 139.

MAURO, V. P. 2018. Codon optimization in the production of recombinant biotherapeutics: potential risks and considerations. *BioDrugs,* 32**,** 69-81.

MAZANEC, M. B., COUDRET, C. L. & FLETCHER, D. R. 1995. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. *J Virol,* 69**,** 1339-43.

MCCOWN, M. F. & PEKOSZ, A. 2005. The influenza A virus M2 cytoplasmic tail is required for infectious virus production and efficient genome packaging. *J Virol,* 79**,** 3595-605.

MCGILL, J., HEUSEL, J. W. & LEGGE, K. L. 2009. Innate immune control and regulation of influenza virus infections. *Journal of leukocyte biology,* 86**,** 803-812.

MCMAHON, M., O'DELL, G., TAN, J., SÁRKÖZY, A., VADOVICS, M., CARREÑO, J. M., PUENTE-MASSAGUER, E., MURAMATSU, H., BAJUSZ, C., RIJNINK, W., BEATTIE, M., TAM, Y. K., KIRKPATRICK ROUBIDOUX, E., FRANCISCO, I., STROHMEIER, S., KANEKIYO, M., GRAHAM, B. S., KRAMMER, F. & PARDI, N. 2022. Assessment of a quadrivalent nucleoside-modified mRNA vaccine that protects against group 2 influenza viruses. *Proceedings of the National Academy of Sciences of the United States of America,* 119**,** e2206333119.

MEDICAGO. 2019. *NEW DRUG SUBMISSION ACCEPTED FOR SCIENTIFIC REVIEW BY HEALTH CANADA* [Online]. Available: https://media.medicago.com/webfolder_download/abfd74e3732457c107f1eab4419f17c4/medicagos-newdrug-asubmission-press-release/6d895df2226d722e3777d7636e98cd72bb47c23e/medicagos-new-drugasubmission-press-release.pdf [Accessed April 4, 2020].

MEDZHITOV, R. & JANEWAY, C. A., JR. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell,* 91**,** 295-8.

MERIÇ, S., GÜMÜŞ, T. & AYAN, A. 2021. Plant-based Vaccines: The Future of Preventive Healthcare? *Botany—Recent Advances and Applications*.

MEYLAN, E., TSCHOPP, J. & KARIN, M. 2006. Intracellular pattern recognition receptors in the host response. *Nature,* 442**,** 39-44.

MOIN, S. M., BOYINGTON, J. C., BOYOGLU-BARNUM, S., GILLESPIE, R. A., CERUTTI, G., CHEUNG, C. S., CAGIGI, A., GALLAGHER, J. R., BRAND, J., PRABHAKARAN, M., TSYBOVSKY, Y., STEPHENS, T., FISHER, B. E., CREANGA, A., ATACA, S., RAWI, R., CORBETT, K. S., CRANK, M. C., KARLSSON HEDESTAM, G. B., GORMAN, J., MCDERMOTT, A. B., HARRIS, A. K., ZHOU, T., KWONG, P. D., SHAPIRO, L., MASCOLA, J. R., GRAHAM, B. S. & KANEKIYO, M. 2022. Co-immunization with hemagglutinin stem immunogens elicits cross-group neutralizing antibodies and broad protection against influenza A viruses. *Immunity,* 55**,** 2405-2418.e7.

MOKOENA, N. B., MOETLHOA, B., RUTKOWSKA, D. A., MAMPUTHA, S., DIBAKWANE, V. S., TSEKOA, T. L. & O'KENNEDY, M. M. 2019. Plant-produced Bluetongue chimaeric VLP vaccine candidates elicit serotype-specific immunity in sheep. *Vaccine,* 37**,** 6068-6075.

MOMOSE, F., BASLER, C. F., O'NEILL, R. E., IWAMATSU, A., PALESE, P. & NAGATA, K. 2001. Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis. *Journal of Virology,* 75**,** 1899-1908.

MOMOSE, F., HANDA, H. & NAGATA, K. 1996. Identification of host factors that regulate the influenza virus RNA polymerase activity. *Biochimie,* 78**,** 1103-1108.

MONNE, I., CATTOLI, G., MAZZACAN, E., AMARIN, N. M., MAAITAH, H. M. A., AL-NATOUR, M. Q. & CAPUA, I. 2007. Genetic comparison of H9N2 AI viruses isolated in Jordan in 2003. *Avian diseases,* 51**,** 451-454.

MONNE, I., HUSSEIN, H. A., FUSARO, A., VALASTRO, V., HAMOUD, M. M., KHALEFA, R. A., DARDIR, S. N., RADWAN, M. I., CAPUA, I. & CATTOLI, G. 2013. H9N2 influenza A virus circulates in H5N1 endemically infected poultry population in Egypt. *Influenza and other respiratory viruses,* 7**,** 240-243.

MONREAL-ESCALANTE, E., RAMOS-VEGA, A., ANGULO, C. & BAÑUELOS-HERNÁNDEZ, B. 2022. Plant-based vaccines: Antigen Design, Diversity, and strategies for high Level production. *Vaccines,* 10**,** 100.

MORADI VAHDAT, M., HEMMATI, F., GHORBANI, A., RUTKOWSKA, D., AFSHARIFAR, A., ESKANDARI, M. H., REZAEI, N. & NIAZI, A. 2021.

Hepatitis B core-based virus-like particles: A platform for vaccine development in plants. *Biotechnology Reports,* 29**,** e00605.

MORRIS, E. J., ERASMUS, C. & O'KENNEDY, M. M. 2016. An African perspective: using the new biosciences to support the African development agenda. *Creating Sustainable Bioeconomies.* Routledge.

MORTIMER, E., MACLEAN, J. M., MBEWANA, S., BUYS, A., WILLIAMSON, A.-L., HITZEROTH, I. I. & RYBICKI, E. P. 2012. Setting up a platform for plant-based influenza virus vaccine production in South Africa. *BMC biotechnology,* 12**,** 1-10.

MOUNTS, A. W., KWONG, H., IZURIETA, H. S., HO, Y.-Y., AU, T.-K., LEE, M., BRIDGES, C. B., WILLIAMS, S. W., MAK, K. H. & KATZ, J. M. 1999. Case-control study of risk factors for avian influenza A (H5N1) disease, Hong Kong, 1997. *The Journal of infectious diseases,* 180**,** 505-508.

MUSIYCHUK, K., STEPHENSON, N., BI, H., FARRANCE, C. E., OROZOVIC, G., BRODELIUS, M., BRODELIUS, P., HORSEY, A., UGULAVA, N. & SHAMLOUL, A. M. 2007. A launch vector for the production of vaccine antigens in plants. *Influenza and other respiratory viruses,* 1**,** 19-25.

NAGUIB, M. M., ARAFA, A.-S. A., EL-KADY, M. F., SELIM, A. A., GUNALAN, V., MAURER-STROH, S., GOLLER, K. V., HASSAN, M. K., BEER, M. & ABDELWHAB, E. 2015. Evolutionary trajectories and diagnostic challenges of potentially zoonotic avian influenza viruses H5N1 and H9N2 co-circulating in Egypt. *J Infection, Genetics Evolution,* 34**,** 278-291.

NAGUIB, M. M., VERHAGEN, J. H., SAMY, A., ERIKSSON, P., FIFE, M., LUNDKVIST, Å., ELLSTRÖM, P. & JÄRHULT, J. D. 2019. Avian influenza viruses at the wild–domestic bird interface in Egypt. *Infection ecology and epidemiology,* 9**,** 1575687.

NAGY, A., METTENLEITER, T. & ABDELWHAB, E. 2017. A brief summary of the epidemiology and genetic relatedness of avian influenza H9N2 virus in birds and mammals in the Middle East and North Africa. *Epidemiology and Infection,* 145**,** 3320-3333.

NAKAMURA, K., IMADA, T., IMAI, K., YAMAMOTO, Y., TANIMURA, N., YAMADA, M., MASE, M., TSUKAMOTO, K. & YAMAGUCHI, S. 2008. Pathology of specific-pathogen-free chickens inoculated with H5N1 avian influenza viruses isolated in Japan in 2004. *Avian Dis,* 52**,** 8-13.

NAKATANI, H., NAKAMURA, K., YAMAMOTO, Y., YAMADA, M. & YAMAMOTO, Y. 2005. Epidemiology, pathology, and immunohistochemistry of layer hens naturally affected with H5N1 highly pathogenic avian influenza in Japan. *Avian diseases,* 49**,** 436-441.

NAO, N., YAMAGISHI, J., MIYAMOTO, H., IGARASHI, M., MANZOOR, R., OHNUMA, A., TSUDA, Y., FURUYAMA, W., SHIGENO, A. & KAJIHARA, M. 2017. Genetic predisposition to acquire a polybasic cleavage site for highly pathogenic avian influenza virus hemagglutinin. *mBio,* 8**,** e02298-16.

NASKALSKA, A. & PYRĆ, K. 2015. Virus Like Particles as Immunogens and Universal Nanocarriers. *Pol J Microbiol,* 64**,** 3-13.

NAYAK, D. P., HUI, E. K. & BARMAN, S. 2004. Assembly and budding of influenza virus. *Virus Res,* 106**,** 147-65.

NAYAK, D. P. & REICHL, U. 2004. Neuraminidase activity assays for monitoring MDCK cell culture derived influenza virus. . *Journal of virological methods.,* 122(1),**,** 9-15.

NEGASH, T., AL-GARIB, S. & GRUYS, E. 2004. Comparison of in ovo and post-hatch vaccination with particular reference to infectious bursal disease. A review. *Veterinary quarterly,* 26**,** 76-87.

NEGOVETICH, N. J., FEEROZ, M. M., JONES-ENGEL, L., WALKER, D., ALAM, S. R., HASAN, K., SEILER, P., FERGUSON, A., FRIEDMAN, K. & BARMAN, S. 2011. Live bird markets of Bangladesh: H9N2 viruses and the near absence of highly pathogenic H5N1 influenza. *PLoS One,* 6**,** e19311.

NEUMANN, G., BROWNLEE, G., FODOR, E. & KAWAOKA, Y. 2004. Orthomyxovirus replication, transcription, and polyadenylation. *Biology of Negative Strand Rna Viruses: the Power of Reverse Genetics.* Springer.

NEUMANN, G., CASTRUCCI, M. R. & KAWAOKA, Y. 1997. Nuclear import and export of influenza virus nucleoprotein. *J Virol,* 71**,** 9690-700.

NEUMANN, G., FUJII, K., KINO, Y. & KAWAOKA, Y. 2005. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. *Proceedings of the National Academy of Sciences,* 102**,** 16825-16829.

NEUMANN, G., HUGHES, M. T. & KAWAOKA, Y. 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *Embo j,* 19**,** 6751-8.

NEUMANN, G., WATANABE, T., ITO, H., WATANABE, S., GOTO, H., GAO, P., HUGHES, M., PEREZ, D. R., DONIS, R. & HOFFMANN, E. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proceedings of the National Academy of Sciences,* 96**,** 9345-9350.

NGUYEN, T.-Q., ROLLON, R. & CHOI, Y.-K. 2021. Animal models for influenza research: strengths and weaknesses. *Viruses,* 13**,** 1011.

NGUYEN, T., LIU, X. K., ZHANG, Y. & DONG, C. 2006. BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation. *The Journal of Immunology,* 176**,** 7354-7360.

NICAS, M., NAZAROFF, W. W. & HUBBARD, A. 2005. Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. *Journal of occupational and environmental hygiene,* 2**,** 143-154.

NICHOL, S. T., PENN, C. R. & MAHY, B. W. 1981. Evidence for the involvement of influenza A (fowl plague Rostock) virus protein P2 in ApG and mRNA primed in vitro RNA synthesis. *Journal of General Virology,* 57**,** 407-413.

NIE, J., LI, Q., WU, J., ZHAO, C., HAO, H., LIU, H., ZHANG, L., NIE, L., QIN, H. & WANG, M. 2020. Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay. *Nature protocols,* 15**,** 3699-3715.

NING, Z. Y., LUO, M. Y., QI, W. B., YU, B., JIAO, P. R. & LIAO, M. 2009. Detection of expression of influenza virus receptors in tissues of BALB/c mice by histochemistry. *Vet Res Commun,* 33**,** 895-903.

NISHIMURA, K., SANO, M., OHTAKA, M., FURUTA, B., UMEMURA, Y., NAKAJIMA, Y., IKEHARA, Y., KOBAYASHI, T., SEGAWA, H. & TAKAYASU, S. 2011. Development of defective and persistent Sendai virus vector a unique gene delivery/expression system ideal for cell reprogramming. *Journal of Biological Chemistry,* 286**,** 4760-4771.

NOAD, R. & ROY, P. 2003. Virus-like particles as immunogens. *Trends in microbiology,* 11**,** 438-444.

NOBUSAWA, E., AOYAMA, T., KATO, H., SUZUKI, Y., TATENO, Y. & NAKAJIMA, K. 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology,* 182**,** 475-485.

NOORAEI, S., BAHRULOLUM, H., HOSEINI, Z. S., KATALANI, C., HAJIZADE, A., EASTON, A. J. & AHMADIAN, G. 2021. Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers. *Journal of Nanobiotechnology,* 19**,** 59.

NORKUNAS, K.-J. 2014. *Development of a transient, high-level expression platform for protein production in plants.* Queensland University of Technology.

NYABOGA, E., TRIPATHI, J. N., MANOHARAN, R. & TRIPATHI, L. 2014. Agrobacterium-mediated genetic transformation of yam (Dioscorea

rotundata): an important tool for functional study of genes and crop improvement. *Frontiers in Plant Science,* 5**,** 463.

O'NEILL, R. E., TALON, J. & PALESE, P. 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *The EMBO journal,* 17**,** 288-296.

OCHI, A., DANESH, A., SENEVIRATNE, C., BANNER, D., DEVRIES, M. E., ROWE, T., XU, L., RAN, L., CZUB, M., BOSINGER, S. E., CAMERON, M. J., CAMERON, C. M. & KELVIN, D. J. 2008. Cloning, expression and immunoassay detection of ferret IFN-gamma. *Dev Comp Immunol,* 32**,** 890-7.

OIE/FAO/IZSVE. 2007. *Vaccination: a tool for the control of avian influenza* [Online]. Verona (Italy): Scientific Conference, co-organised and supported by European Union Available: https://www.oie.int/doc/ged/D4410.PDF [Accessed March 22 2020].

PALESE, P. 2007. Orthomyxoviridae: the viruses and their replication. *Fields virology***,** 1647-1689.

PALESE, P. & SHAW, M. 2006. In Howley, PM (ed), Fields Virology, .

PANG, I. K. & IWASAKI, A. 2011. Inflammasomes as mediators of immunity against influenza virus. *Trends in immunology,* 32**,** 34-41.

PAPPAS, C., VISWANATHAN, K., CHANDRASEKARAN, A., RAMAN, R., KATZ, J. M., SASISEKHARAN, R. & TUMPEY, T. M. 2010. Receptor specificity and transmission of H2N2 subtype viruses isolated from the pandemic of 1957. *PLoS One,* 5**,** e11158.

PARIJA, S. C. 2014. *Textbook of Microbiology & Immunology*
India, Elsevier.

PARK, M.-S., STEEL, J., GARCÍA-SASTRE, A., SWAYNE, D. & PALESE, P. 2006. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. *Proceedings of the National Academy of Sciences,* 103**,** 8203-8208.

PARK, W.-J., YOU, S.-H., CHOI, H.-A., CHU, Y.-J. & KIM, G.-J. 2015. Over-expression of recombinant proteins with N-terminal His-tag via subcellular uneven distribution in Escherichia coli. *Acta Biochim Biophys Sin,* 47**,** 488-495.

PARK, Y. W. & KATZE, M. G. 1995. Translational Control by Influenza Virus identification of CIS-acting sequences and trans-acting factors which may regulate selective viral mRNA translation. *Journal of Biological Chemistry,* 270**,** 28433-28439.

PASICK, J., HANDEL, K., ROBINSON, J., COPPS, J., RIDD, D., HILLS, K., KEHLER, H., COTTAM-BIRT, C., NEUFELD, J. & BERHANE, Y. 2005. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. *Journal of General Virology,* 86**,** 727-731.

PATRICK, J. V. & RICHARD, B. T. 2003. Review of Rapid Diagnostic Tests for Influenza. . *Clinical and Applied Immunology,* 4**,** 151-172.

PAWAR, K. R. & WAGHMARE, S. G. 2016. Agroinfiltration-Effective Method of Gene Transfer.

PEABODY, J., MUTTIL, P., CHACKERIAN, B. & TUMBAN, E. 2017. Characterization of a spray-dried candidate HPV L2-VLP vaccine stored for multiple years at room temperature. *Papillomavirus Research,* 3**,** 116-120.

PEACEY, M., WILSON, S., BAIRD, M. A. & WARD, V. K. 2007. Versatile RHDV virus-like particles: Incorporation of antigens by genetic modification and chemical conjugation. *Biotechnology and bioengineering,* 98**,** 968-977.

PEACOCK, T. P., JAMES, J., SEALY, J. E. & IQBAL, M. 2019. A global perspective on H9N2 avian influenza virus. *Viruses,* 11**,** 620.

PECETTA, S. & RAPPUOLI, R. 2022. mRNA, the beginning of a new influenza vaccine game. *Proceedings of the National Academy of Sciences,* 119**,** e2217533119.

PÊRA, F. F., MUTEPFA, D. L., KHAN, A. M., ELS, J. H., MBEWANA, S., VAN DIJK, A. A., RYBICKI, E. P. & HITZEROTH, I. I. 2015. Engineering and expression of a human rotavirus candidate vaccine in Nicotiana benthamiana. *Virology journal,* 12**,** 1-11.

PEREIRA, H., TŮMOVÁ, B. & LAW, V. 1965. Avian influenza A viruses. *Bulletin of the World Health Organization,* 32**,** 855.

PERKINS, L. E. L. 2001. *The pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in avian and mammalian species.* uga.

PEYRET, H., GEHIN, A., THUENEMANN, E. C., BLOND, D., EL TURABI, A., BEALES, L., CLARKE, D., GILBERT, R. J., FRY, E. E. & STUART, D. I. 2015. Tandem fusion of hepatitis B core antigen allows assembly of virus-like particles in bacteria and plants with enhanced capacity to accommodate foreign proteins. *PLoS One,* 10**,** e0120751.

PEYRET, H. & LOMONOSSOFF, G. P. 2013. The pEAQ vector series: the easy and quick way to produce recombinant proteins in plants. *Plant Mol Biol,* 83**,** 51-8.

PEYRET, H., PONNDORF, D., MESHCHERIAKOVA, Y., RICHARDSON, J. & LOMONOSSOFF, G. P. 2020. Covalent protein display on Hepatitis B core-like particles in plants through the in vivo use of the SpyTag/SpyCatcher system. *Scientific Reports,* 10**,** 17095.

PEYRET, H., STEELE, J. F., JUNG, J.-W., THUENEMANN, E. C., MESHCHERIAKOVA, Y. & LOMONOSSOFF, G. P. 2021. Producing vaccines against enveloped viruses in plants: Making the impossible, difficult. *Vaccines,* 9**,** 780.

PHAN, H. T., GRESCH, U. & CONRAD, U. 2018. In vitro-formulated oligomers of strep-tagged avian influenza haemagglutinin produced in plants cause neutralizing immune responses. *Frontiers in bioengineering and biotechnology,* 6**,** 115.

PHAN, H. T., PHAM, V. T., HO, T. T., PHAM, N. B., CHU, H. H., VU, T. H., ABDELWHAB, E. M., SCHEIBNER, D., METTENLEITER, T. C. & HANH, T. X. 2020. Immunization with plant-derived multimeric H5 hemagglutinins protect chicken against highly pathogenic avian influenza virus H5N1. *Vaccines,* 8**,** 593.

PILLAI, S. P. & LEE, C. W. 2010. Species and age related differences in the type and distribution of influenza virus receptors in different tissues of chickens, ducks and turkeys. *Virol J,* 7**,** 5.

PILLET, S., AUBIN, É., TRÉPANIER, S., BUSSIÈRE, D., DARGIS, M., POULIN, J.-F., YASSINE-DIAB, B., WARD, B. J. & LANDRY, N. 2016. A plant-derived quadrivalent virus like particle influenza vaccine induces cross-reactive antibody and T cell response in healthy adults. *Clinical immunology,* 168**,** 72-87.

PILLET, S., AUBIN, É., TRÉPANIER, S., POULIN, J.-F., YASSINE-DIAB, B., TER MEULEN, J., WARD, B. J. & LANDRY, N. 2018. Humoral and cell-mediated immune responses to H5N1 plant-made virus-like particle vaccine are differentially impacted by alum and GLA-SE adjuvants in a Phase 2 clinical trial. *npj Vaccines,* 3**,** 1-9.

PILLET, S., RACINE, T., NFON, C., DI LENARDO, T., BABIUK, S., WARD, B., KOBINGER, G. & LANDRY, N. 2015. Plant-derived H7 VLP vaccine elicits protective immune response against H7N9 influenza virus in mice and ferrets. *Vaccine,* 33**,** 6282-6289.

PINTO, L. H. & LAMB, R. A. 2006. The M2 proton channels of influenza A and B viruses. *Journal of Biological Chemistry,* 281**,** 8997-9000.

PITISUTTITHUM, P., BOONNAK, K., CHAMNANCHANUNT, S., PUTHAVATHANA, P., LUVIRA, V., LERDSAMRAN, H., KAEWKUNGWAL, J., LAWPOOLSRI, S., THANACHARTWET, V. & SILACHAMROON, U.

2017. Safety and immunogenicity of a live attenuated influenza H5 candidate vaccine strain A/17/turkey/Turkey/05/133 H5N2 and its priming effects for potential pre-pandemic use: a randomised, double-blind, placebo-controlled trial. *The Lancet Infectious Diseases,* 17**,** 833-842.

POHLMANN, A., STARICK, E., HARDER, T., GRUND, C., HÖPER, D., GLOBIG, A., STAUBACH, C., DIETZE, K., STREBELOW, G. & ULRICH, R. G. 2017. Outbreaks among wild birds and domestic poultry caused by reassorted influenza A (H5N8) clade 2.3. 4.4 viruses, Germany, 2016. *Emerging Infectious Diseases,* 23**,** 633.

PU, J., SUN, H., QU, Y., WANG, C., GAO, W., ZHU, J., SUN, Y., BI, Y., HUANG, Y. & CHANG, K.-C. 2017. M gene reassortment in H9N2 influenza virus promotes early infection and replication: contribution to rising virus prevalence in chickens in China. *Journal of virology,* 91**,** e02055-16.

PU, J., WANG, S., YIN, Y., ZHANG, G., CARTER, R. A., WANG, J., XU, G., SUN, H., WANG, M. & WEN, C. 2015. Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. *Proceedings of the National Academy of Sciences,* 112**,** 548-553.

PUA, T.-L., CHAN, X. Y., LOH, H.-S., OMAR, A. R., YUSIBOV, V., MUSIYCHUK, K., HALL, A. C., COFFIN, M. V., SHOJI, Y. & CHICHESTER, J. A. 2017. Purification and immunogenicity of hemagglutinin from highly pathogenic avian influenza virus H5N1 expressed in Nicotiana benthamiana. *Human vaccines & immunotherapeutics,* 13**,** 306-313.

PULENDRAN, B., P, S. A. & O'HAGAN, D. T. 2021. Emerging concepts in the science of vaccine adjuvants. *Nature Reviews Drug Discovery,* 20**,** 454-475.

PUMPENS, P. & GRENS, E. 2001. HBV core particles as a carrier for B cell/T cell epitopes. *Intervirology,* 44**,** 98-114.

PUSCH, E. A. & SUAREZ, D. L. 2018. The multifaceted zoonotic risk of H9N2 avian influenza. *Veterinary sciences,* 5**,** 82.

PYANKOVA, O. G., SUSLOPAROV, I. M., MOISEEVA, A. A., KOLOSOVA, N. P., ONKHONOVA, G. S., DANILENKO, A. V., VAKALOVA, E. V., SHENDO, G. L., NEKESHINA, N. N. & NOSKOVA, L. N. 2021. Isolation of clade 2.3. 4.4 b A (H5N8), a highly pathogenic avian influenza virus, from a worker during an outbreak on a poultry farm, Russia, December 2020. *Eurosurveillance,* 26**,** 2100439.

RACANIELLO, V. R. & PALESE, P. 1979. Influenza B virus genome: assignment of viral polypeptides to RNA segments. *Journal of Virology,* 29**,** 361-373.

RAJARAM, S., BOIKOS, C., GELONE, D. K. & GANDHI, A. 2020. Influenza vaccines: the potential benefits of cell-culture isolation and manufacturing. *Therapeutic Advances in Vaccines and Immunotherapy,* 8**,** 2515135520908121.

RAO, S., KONG, W.-P., WEI, C.-J., YANG, Z.-Y., NASON, M., STYLES, D., DETOLLA, L. J., SORRELL, E. M., SONG, H. & WAN, H. 2008. Multivalent HA DNA vaccination protects against highly pathogenic H5N1 avian influenza infection in chickens and mice. *PLoS One,* 3.

REN, Z., JI, X., MENG, L., WEI, Y., WANG, T., FENG, N., ZHENG, X., WANG, H., LI, N. & GAO, X. 2015. H5N1 influenza virus-like particle vaccine protects mice from heterologous virus challenge better than whole inactivated virus. *Virus research,* 200**,** 9-18.

RESA-INFANTE, P., JORBA, N., ZAMARREÑO, N., FERNÁNDEZ, Y., JUÁREZ, S. & ORTÍN, J. 2008. The host-dependent interaction of α-importins with influenza PB2 polymerase subunit is required for virus RNA replication. *PloS one,* 3.

ROBB, N. C., SMITH, M., VREEDE, F. T. & FODOR, E. 2009. NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome. *Journal of general virology,* 90**,** 1398-1407.

RODRIGUEZ, L., NOGALES, A. & MARTÍNEZ-SOBRIDO, L. 2017. Influenza A Virus Studies in a Mouse Model of Infection. *Journal of Visualized Experiments*.

ROGERS, G. N. & D'SOUZA, B. L. 1989. Receptor binding properties of human and animal H1 influenza virus isolates. *Virology,* 173**,** 317-322.

ROGERS, G. N. & PAULSON, J. C. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology,* 127**,** 361-373.

ROTT, R. J. 1980. Genetic determinants for infectivity and pathogenicity of influenza viruses. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences,* 288**,** 393-399.

ROY, A.-M. M., PARKER, J. S., PARRISH, C. R. & WHITTAKER, G. R. 2000. Early stages of influenza virus entry into Mv-1 lung cells: involvement of dynamin. *Virology,* 267**,** 17-28.

RUIGROK, R. W., BARGE, A., DURRER, P., BRUNNER, J., MA, K. & WHITTAKER, G. R. 2000. Membrane interaction of influenza virus M1 protein. *Virology,* 267**,** 289-98.

RUSSELL, C. J. 2021. Hemagglutinin Stability and Its Impact on Influenza A Virus Infectivity, Pathogenicity, and Transmissibility in Avians, Mice, Swine, Seals, Ferrets, and Humans. *Viruses,* 13**,** 746.

RUST, M. J., LAKADAMYALI, M., ZHANG, F. & ZHUANG, X. 2004. Assembly of endocytic machinery around individual influenza viruses during viral entry. *Nature structural and molecular biology,* 11**,** 567-573.

SAELENS, X., VANLANDSCHOOT, P., MARTINET, W., MARAS, M., NEIRYNCK, S., CONTRERAS, R., FIERS, W. & JOU, W. M. 1999. Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. *European journal of biochemistry,* 260**,** 166-175.

SAINSBURY, F. & LOMONOSSOFF, G. P. 2008. Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant physiology,* 148**,** 1212-1218.

SAINSBURY, F., THUENEMANN, E. C. & LOMONOSSOFF, G. P. 2009. pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol J,* 7**,** 682-93.

SAKAI, T., NISHIMURA, S. I., NAITO, T. & SAITO, M. 2017. Influenza A virus hemagglutinin and neuraminidase act as novel motile machinery. *Scientific reports,* 7**,** 45043.

SALAHELDIN, A. H., ELBESTAWY, A. R., ABDELKADER, A. M., SULTAN, H. A., IBRAHIM, A. A., ABD EL-HAMID, H. S. & ABDELWHAB, E. M. 2022. Isolation of Genetically Diverse H5N8 Avian Influenza Viruses in Poultry in Egypt, 2019–2021. *Viruses,* 14**,** 1431.

SAMY, A. & NAGUIB, M. M. 2018. Avian respiratory coinfection and impact on avian influenza pathogenicity in domestic poultry: field and experimental findings. *Veterinary sciences,* 5**,** 23.

SASAI, M. & YAMAMOTO, M. 2013. Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors. *Int Rev Immunol,* 32**,** 116-33.

SAWOO, O., DUBLINEAU, A., BATÉJAT, C., ZHOU, P., MANUGUERRA, J.-C. & LECLERCQ, I. 2014. Cleavage of hemagglutinin-bearing lentiviral pseudotypes and their use in the study of influenza virus persistence. *PLoS One,* 9**,** e106192.

SCHÄDLER, J., SIGRIST, B., MEIER, S. M., ALBINI, S. & WOLFRUM, N. 2019. Virus-like particles in a new vaccination approach against infectious laryngotracheitis. *Journal of General Virology,* 100**,** 1013-1026.

SCHEIBNER, D., BREITHAUPT, A., LUTTERMANN, C., BLAUROCK, C., METTENLEITER, T. C. & ABDELWHAB, E. M. 2022. Genetic Determinants for Virulence and Transmission of the Panzootic Avian Influenza Virus H5N8 Clade 2.3. 4.4 in Pekin Ducks. *Journal of Virology***,** e00149-22.

SCHEIFFELE, P., ROTH, M. G. & SIMONS, K. 1997. Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. *Embo j,* 16**,** 5501-8.

SCHNEIDER, J. & WOLFF, T. 2009. Nuclear functions of the influenza A and B viruses NS1 proteins: do they play a role in viral mRNA export? *Vaccine,* 27**,** 6312-6316.

SCOIZEC, A., NIQUEUX, E., THOMAS, R., DANIEL, P., SCHMITZ, A. & LE BOUQUIN, S. 2018. Airborne detection of H5N8 highly pathogenic avian influenza virus genome in poultry farms, France. *Frontiers in Veterinary Science,* 5**,** 15.

SCOLARI, S., ENGEL, S., KREBS, N., PLAZZO, A. P., DE ALMEIDA, R. F., PRIETO, M., VEIT, M. & HERRMANN, A. 2009. Lateral distribution of the transmembrane domain of influenza virus hemagglutinin revealed by time-resolved fluorescence imaging. *J Biol Chem,* 284**,** 15708-16.

SCOTT, S., MOLESTI, E., TEMPERTON, N., FERRARA, F., BÖTTCHER-FRIEBERTSHÄUSER, E. & DALY, J. 2012. The use of equine influenza pseudotypes for serological screening. *J Mol Genet Med,* 6**,** 304-8.

SEDERDAHL, B. K. & WILLIAMS, J. V. 2020. Epidemiology and clinical characteristics of influenza C virus. *Viruses,* 12**,** 89.

SEO, S. H. & WEBSTER, R. G. 2001. Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. *J Virol,* 75**,** 2516-25.

SEPPIC. *Montanide™ range for veterinary vaccines* [Online]. Available: https://www.seppic.com/en/animal-health/montanide [Accessed March, 6 2023].

SHAMLOUL, M., TRUSA, J., METT, V. & YUSIBOV, V. 2014. Optimization and utilization of Agrobacterium-mediated transient protein production in Nicotiana. *JoVE (Journal of Visualized Experiments)***,** e51204.

SHELTON, H., AYORA-TALAVERA, G., REN, J., LOUREIRO, S., PICKLES, R. J., BARCLAY, W. S. & JONES, I. M. 2011. Receptor binding profiles of avian influenza virus hemagglutinin subtypes on human cells as a predictor of pandemic potential. *J Virol,* 85**,** 1875-80.

SHINYA, K., EBINA, M., YAMADA, S., ONO, M., KASAI, N. & KAWAOKA, Y. 2006. Avian flu: influenza virus receptors in the human airway. *Nature,* 440**,** 435-6.

SHOPE, R. E. 1931. Swine influenza: I. Experimental transmission and pathology. *The Journal of experimental medicine,* 54**,** 349-359.

SHORS, T. 2016. *Understanding viruses*, Jones & Bartlett Publishers.

SHORTRIDGE, K. Pandemic influenza: a zoonosis? Seminars in respiratory infections, 1992. 11-25.

SHRESTHA, S., FOXMAN, B., BERUS, J., VAN PANHUIS, W. G., STEINER, C., VIBOUD, C. & ROHANI, P. 2015. The role of influenza in the epidemiology of pneumonia. *Scientific reports,* 5**,** 15314.

SINGH, A., RAJPUT, V., SINGH, A. K., SENGAR, R. S., SINGH, R. K. & MINKINA, T. 2021. Transformation techniques and their role in crop improvements: a global scenario of GM crops. *Policy Issues in Genetically Modified Crops.* Elsevier.

SLEMAN, S. 2017. How Influenza a causes ‘‘Epidemics and Pandemics’’among the population: novel targets for anti-influenza molecules. *Journal of Infectious Diseases and Epidemiology,* 1**,** 00005.

SLEMONS, R. D., JOHNSON, D. C., OSBORN, J. S. & HAYES, F. 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. *Avian diseases***,** 119-124.

SLOMKA, M. J., PURANIK, A., MAHMOOD, S., THOMAS, S. S., SEEKINGS, A. H., BYRNE, A. M., NÚÑEZ, A., BIANCO, C., MOLLETT, B. C. & WATSON, S. 2019. Ducks are susceptible to infection with a range of doses of H5N8 highly pathogenic avian influenza virus (2016, Clade 2.3. 4.4 b) and are largely resistant to virus-specific mortality, but efficiently transmit infection to contact Turkeys. *Avian diseases,* 63**,** 172-180.

SMITH, G. L. 2001. *New challenges to health: the threat of virus infection*, Cambridge University Press.

SMITH, J. G., LIU, X., KAUFHOLD, R. M., CLAIR, J. & CAULFIELD, M. J. 2001. Development and validation of a gamma interferon ELISPOT assay for quantitation of cellular immune responses to varicella-zoster virus. *Clin Diagn Lab Immunol,* 8**,** 871-9.

SMITH, L. R., WLOCH, M. K., YE, M., REYES, L. R., BOUTSABOUALOY, S., DUNNE, C. E., CHAPLIN, J. A., RUSALOV, D., ROLLAND, A. P. & FISHER, C. L. 2010. Phase 1 clinical trials of the safety and immunogenicity

of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin. *Vaccine,* 28**,** 2565-2572.

SMITH, T., O'KENNEDY, M. M., WANDRAG, D. B., ADEYEMI, M. & ABOLNIK, C. 2020. Efficacy of a plant-produced virus-like particle vaccine in chickens challenged with Influenza A H6N2 virus. *Plant biotechnology journal,* 18**,** 502-512.

SMITH, W., ANDREWES, C. H. & LAIDLAW, P. P. 1933. A virus obtained from influenza patients. *Lancet***,** 66-8.

SODA, K., ASAKURA, S., OKAMATSU, M. & SAKODA, Y. 2011. H9N2 influenza virus acquires intravenous pathogenicity on the introduction of a pair of di-basic amino acid residues at the cleavage site of the hemagglutinin and consecutive passages in chickens. *Virology journal,* 8**,** 64.

SOGHOIAN, D. Z. & STREECK, H. 2010. Cytolytic CD4(+) T cells in viral immunity. *Expert Rev Vaccines,* 9**,** 1453-63.

SONG, H., WAN, H., ARAYA, Y. & PEREZ, D. R. 2009. Partial direct contact transmission in ferrets of a mallard H7N3 influenza virus with typical avian-like receptor specificity. *Virol J,* 6**,** 126.

SOUVESTRE, M., GUINAT, C., NIQUEUX, E., ROBERTET, L., CROVILLE, G., PAUL, M., SCHMITZ, A., BRONNER, A., ETERRADOSSI, N. & GUÉRIN, J.-L. 2019. Role of backyard flocks in transmission dynamics of highly pathogenic avian influenza A (H5N8) Clade 2.3. 4.4, France, 2016–2017. *Emerging infectious diseases,* 25**,** 551.

SPACKMAN, E. & SUAREZ, D. L. 2008. Type A influenza virus detection and quantitation by real-time RT-PCR. *Avian influenza virus.* Springer.

SPACKMAN, E. & SWAYNE, D. E. 2013. Vaccination of gallinaceous poultry for H5N1 highly pathogenic avian influenza: current questions and new technology. *Virus research,* 178**,** 121-132.

SPITSIN, S., ANDRIANOV, V., POGREBNYAK, N., SMIRNOV, Y., BORISJUK, N., PORTOCARRERO, C., VEGUILLA, V., KOPROWSKI, H. & GOLOVKIN, M. 2009. Immunological assessment of plant-derived avian flu H5/HA1 variants. *Vaccine,* 27**,** 1289-1292.

STAHL, S., MACKAY, P., MAGAZIN, M., BRUCE, S. A. & MURRAY, K. 1982. Hepatitis B virus core antigen: synthesis in Escherichia coli and application in diagnosis. *Proceedings of the National Academy of Sciences,* 79**,** 1606-1610.

STALLKNECHT, D. E. 2003. Ecology and epidemiology of avian influenza viruses in wild bird populations: waterfowl, shorebirds, pelicans, cormorants, etc. *Avian diseases,* 47**,** 61-69.

STEEG, P., MOORE, R., JOHNSON, H. & OPPENHEIM, J. 1982. Regulation of murine macrophage Ia antigen expression by a lymphokine with immune interferon activity. *The Journal of experimental medicine,* 156**,** 1780-1793.

STEPHENSON, I., NICHOLSON, K. G., GLÜCK, R., MISCHLER, R., NEWMAN, R. W., PALACHE, A. M., VERLANDER, N. Q., WARBURTON, F., WOOD, J. M. & ZAMBON, M. C. 2003. Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. *The Lancet,* 362**,** 1959-1966.

STIENEKE-GRÖBER, A., VEY, M., ANGLIKER, H., SHAW, E., THOMAS, G., ROBERTS, C., KLENK, H. D. & GARTEN, W. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *Embo j,* 11**,** 2407-14.

STRASSER, R., STADLMANN, J., SCHÄHS, M., STIEGLER, G., QUENDLER, H., MACH, L., GLÖSSL, J., WETERINGS, K., PABST, M. & STEINKELLNER, H. 2008. Generation of glyco-engineered Nicotiana benthamiana for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant biotechnology journal,* 6**,** 392-402.

STURM-RAMIREZ, K., HULSE-POST, D., GOVORKOVA, E., HUMBERD, J., SEILER, P., PUTHAVATHANA, P., BURANATHAI, C., NGUYEN, T., CHAISINGH, A. & LONG, H. 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? *Journal of virology,* 79**,** 11269-11279.

SUAREZ, D. & SCHULTZ-CHERRY, S. 2000a. Immunology of avian influenza virus: a review. *Developmental & Comparative Immunology,* 24**,** 269-283.

SUAREZ, D. & SENNE, D. 2000. The link between the live bird markets and the Pennsylvania H5N2 avian influenza outbreak of 1983-84. *Avian diseases*.

SUAREZ, D. L., GARCIA, M., LATIMER, J., SENNE, D. & PERDUE, M. 1999. Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. *Journal of virology,* 73**,** 3567-3573.

SUAREZ, D. L. & SCHULTZ-CHERRY, S. 2000b. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. *Avian diseases***,** 861-868.

SUAREZ, D. L., SENNE, D. A., BANKS, J., BROWN, I. H., ESSEN, S. C., LEE, C.-W., MANVELL, R. J., MATHIEU-BENSON, C., MORENO, V. & PEDERSEN, J. C. 2004. Recombination resulting in virulence shift in avian influenza outbreak, Chile. *Emerging infectious diseases,* 10**,** 693.

SUGIYAMA, K., KAMADA, T., SHIMIZU, K. & WATANABE, Y. 1976. Preferential phosphorylation of NP-protein of influenza A2 virus by virion-associated protein kinase. *Jpn J Microbiol,* 20**,** 227-32.

SUGUITAN JR, A. L., MCAULIFFE, J., MILLS, K. L., JIN, H., DUKE, G., LU, B., LUKE, C. J., MURPHY, B., SWAYNE, D. E. & KEMBLE, G. 2006. Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS medicine,* 3.

SUN, X., BELSER, J. A., PULIT-PENALOZA, J. A., CREAGER, H. M., GUO, Z., JEFFERSON, S. N., LIU, F., YORK, I. A., STEVENS, J. & MAINES, T. R. 2017. Stockpiled pre-pandemic H5N1 influenza virus vaccines with AS03 adjuvant provide cross-protection from H5N2 clade 2.3. 4.4 virus challenge in ferrets. *Virology,* 508**,** 164-169.

SUN, Y., TAN, Y., WEI, K., SUN, H., SHI, Y., PU, J., YANG, H., GAO, G. F., YIN, Y. & FENG, W. 2013. Amino acid 316 of hemagglutinin and the neuraminidase stalk length influence virulence of H9N2 influenza virus in chickens and mice. *Journal of virology,* 87**,** 2963-2968.

SVENSSON, J., ANDERSSON, C., RESELAND, J. E., LYNGSTADAAS, P. & BÜLOW, L. 2006. Histidine tag fusion increases expression levels of active recombinant amelogenin in Escherichia coli. *Protein expression and purification,* 48**,** 134-141.

SWAYNE, D., BECK, J., GARCIA, M. & STONE, H. 1999. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. *Avian Pathology,* 28**,** 245-255.

SWAYNE, D., SUAREZ, D., SCHULTZ-CHERRY, S., TUMPEY, T., KING, D., NAKAYA, T., PALESE, P. & GARCIA-SASTRE, A. 2003. Recombinant paramyxovirus type 1-avian influenza-H7 virus as a vaccine for protection of chickens against influenza and Newcastle disease. *Avian diseases,* 47**,** 1047-1050.

SWAYNE, D. E. 2007. Understanding the complex pathobiology of high pathogenicity avian influenza viruses in birds. *Avian diseases,* 51**,** 242-249.

SWAYNE, D. E. 2009. Avian influenza vaccines and therapies for poultry. *Comparative immunology, microbiology and infectious diseases,* 32**,** 351-363.

SWAYNE, D. E. 2019. *Avian Influenza* [Online]. Southeast Poultry Research Laboratory. Available: https://www.msdvetmanual.com/poultry/avian-influenza/avian-influenza [Accessed 31 August 2022].

SWAYNE, D. E. & KAPCZYNSKI, D. 2008. Strategies and challenges for eliciting immunity against avian influenza virus in birds. *Immunol Rev,* 225**,** 314-31.

SWAYNE, D. E. & KAPCZYNSKI, D. R. 2016. Vaccines and vaccination for avian influenza in poultry. *Animal influenza***,** 378-434.

SWAYNE, D. E., PAVADE, G., HAMILTON, K., VALLAT, B. & MIYAGISHIMA, K. 2011. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. *Rev Sci Tech,* 30**,** 839-70.

SWAYNE, D. E. & SPACKMAN, E. 2013. Current status and future needs in diagnostics and vaccines for high pathogenicity avian influenza. *Dev Biol (Basel),* 135**,** 79-94.

TAKEDA, M., LESER, G. P., RUSSELL, C. J. & LAMB, R. A. 2003. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc Natl Acad Sci U S A,* 100**,** 14610-7.

TAKEYAMA, N., KIYONO, H. & YUKI, Y. 2015. Plant-based vaccines for animals and humans: recent advances in technology and clinical trials. *Therapeutic advances in vaccines,* 3**,** 139-154.

TALAAT, K. R., KARRON, R. A., CALLAHAN, K. A., LUKE, C. J., DILORENZO, S. C., CHEN, G. L., LAMIRANDE, E. W., JIN, H., COELINGH, K. L. & MURPHY, B. R. 2009. A live attenuated H7N3 influenza virus vaccine is well tolerated and immunogenic in a Phase I trial in healthy adults. *Vaccine,* 27**,** 3744-3753.

TANGUAY, S. & KILLION, J. J. 1994. Direct comparison of ELISPOT and ELISA-based assays for detection of individual cytokine-secreting cells. *Lymphokine and cytokine research,* 13**,** 259-263.

TARASENKO, I., TARANOV, A., FIRSOV, A. & DOLGOV, S. 2013. Expression of the nucleotide sequence for the M2e peptide of avian influenza virus in transgenic tobacco plants. *Applied biochemistry and microbiology,* 49**,** 695-701.

TARIQ, H., BATOOL, S., ASIF, S., ALI, M. & ABBASI, B. H. 2021. Virus-Like Particles: Revolutionary Platforms for Developing Vaccines Against Emerging Infectious Diseases. *Front Microbiol,* 12**,** 790121.

TAYLOR, J., WEINBERG, R., KAWAOKA, Y., WEBSTER, R. G. & PAOLETTI, E. 1988. Protective immunity against avian influenza induced by a fowlpox virus recombinant. *Vaccine,* 6**,** 504-508.

TELESHOVA, N., FRANK, I. & POPE, M. 2003. Immunodeficiency virus exploitation of dendritic cells in the early steps of infection. *J Leukoc Biol,* 74**,** 683-90.

TELLIER, R. 2006. Review of aerosol transmission of influenza A virus. *Emerging infectious diseases,* 12**,** 1657.

THI PHAM, V., THI HO, T., TRONG PHAN, H., LE, T. H., BICH PHAM, N., CONRAD, U., HUYEN VU, T. & HOANG CHU, H. 2019. A plant-based artificial haemagglutinin (A/H5N1) strongly induced neutralizing immune responses in mice. *Applied Sciences,* 9**,** 4605.

THOMAS, H. E., PARKER, J. L., SCHREIBER, R. D. & KAY, T. 1998. IFN-gamma action on pancreatic beta cells causes class I MHC upregulation but not diabetes. *The Journal of clinical investigation,* 102**,** 1249-1257.

TIAN, G., ZHANG, S., LI, Y., BU, Z., LIU, P., ZHOU, J., LI, C., SHI, J., YU, K. & CHEN, H. 2005. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. *Virology,* 341**,** 153-162.

TITERMAX®. *TiterMax® Gold - A New and Improved Research Adjuvant* [Online]. Available: http://www.titermax.com/Animal-Testing-Norcross-GA.html [Accessed March, 6 2023].

TOMBARI, W., NSIRI, J., LARBI, I., GUERIN, J. L. & GHRAM, A. 2011. Genetic evolution of low pathogenecity H9N2 Avian influenza viruses in Tunisia: acquisition of new mutations. *Virology Journal,* 8**,** 1-12.

TONG, S., LI, Y., RIVAILLER, P., CONRARDY, C., CASTILLO, D. A. A., CHEN, L.-M., RECUENCO, S., ELLISON, J. A., DAVIS, C. T. & YORK, I. A. 2012. A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences,* 109**,** 4269-4274.

TONG, S., ZHU, X., LI, Y., SHI, M., ZHANG, J., BOURGEOIS, M., YANG, H., CHEN, X., RECUENCO, S. & GOMEZ, J. 2013. New world bats harbor diverse influenza A viruses. *PLoS pathogens,* 9**,** e1003657.

TOSH, C., NAGARAJAN, S., BEHERA, P., RAJUKUMAR, K., PUROHIT, K., KAMAL, R., MURUGKAR, H., GOUNALAN, S., PATTNAIK, B. &

VANAMAYYA, P. 2008. Genetic analysis of H9N2 avian influenza viruses isolated from India. *Archives of virology,* 153**,** 1433-1439.

TRAN, T. 2004. Nguyen, vVC; Pham, TS; Vo, CD; Le, TQM; Ngo, TT; et al. Avian influenza A (H5N1) in 10 patients in Vietnam. *N. Engl. J. Med,* 350**,** 1179-1188.

TREGONING, J. S., RUSSELL, R. F. & KINNEAR, E. 2018. Adjuvanted influenza vaccines. *Human Vaccines & Immunotherapeutics,* 14**,** 550-564.

TSAI, C., CAILLET, C., HU, H., ZHOU, F., DING, H., ZHANG, G., ZHOU, B., WANG, S., LU, S., BUCHY, P., DEUBEL, V., VOGEL, F. R. & ZHOU, P. 2009. Measurement of neutralizing antibody responses against H5N1 clades in immunized mice and ferrets using pseudotypes expressing influenza hemagglutinin and neuraminidase. *Vaccine,* 27**,** 6777-90.

TSE, L. V., HAMILTON, A. M., FRILING, T. & WHITTAKER, G. R. 2014. A novel activation mechanism of avian influenza virus H9N2 by furin. *Journal of virology,* 88**,** 1673-1683.

TSUKAMOTO, K., IMADA, T., TANIMURA, N., OKAMATSU, M., MASE, M., MIZUHARA, T., SWAYNE, D. & YAMAGUCHI, S. 2007. Impact of different husbandry conditions on contact and airborne transmission of H5N1 highly pathogenic avian influenza virus to chickens. *Avian Diseases,* 51**,** 129-132.

TUMPEY, T. M., MAINES, T. R., VAN HOEVEN, N., GLASER, L., SOLÓRZANO, A., PAPPAS, C., COX, N. J., SWAYNE, D. E., PALESE, P. & KATZ, J. M. 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science,* 315**,** 655-659.

UEMATSU, S. & AKIRA, S. 2007. Toll-like receptors and Type I interferons. *J Biol Chem,* 282**,** 15319-23.

ULMANEN, I., BRONI, B. A. & KRUG, R. M. 1981. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription. *Proceedings of the National Academy of Sciences,* 78**,** 7355-7359.

UNGCHUSAK, K., AUEWARAKUL, P., DOWELL, S. F., KITPHATI, R., AUWANIT, W., PUTHAVATHANA, P., UIPRASERTKUL, M., BOONNAK, K., PITTAYAWONGANON, C. & COX, N. J. 2005. Probable person-to-person transmission of avian influenza A (H5N1). *New England Journal of Medicine,* 352**,** 333-340.

VACHER, G. L., KAESER, M. D., MOSER, C., GURNY, R. & BORCHARD, G. 2013. Recent advances in mucosal immunization using virus-like particles. *Molecular pharmaceutics,* 10**,** 1596-1609.

VAN DE SANDT, C. E., KREIJTZ, J. H. & RIMMELZWAAN, G. F. 2012. Evasion of influenza A viruses from innate and adaptive immune responses. *Viruses,* 4**,** 1438-1476.

VAN, P. T., HUONG, H. T., GIANG, N. T., NGOC, P. B., TRANG, V. H., HOANG, P. T., HANH, T. X., CONRAD, U. & HA, C. H. 2020. Protection of chickens against A/H5N1 virus by plant-based haemagglutinin fused IGMFC. *Vietnam Journal of Biotechnology,* 18**,** 477-485.

VEITS, J., LÜSCHOW, D., KINDERMANN, K., WERNER, O., TEIFKE, J. P., METTENLEITER, T. C. & FUCHS, W. 2003. Deletion of the non-essential UL0 gene of infectious laryngotracheitis (ILT) virus leads to attenuation in chickens, and UL0 mutants expressing influenza virus haemagglutinin (H7) protect against ILT and fowl plague. *Journal of general virology,* 84**,** 3343-3352.

VEITS, J., WIESNER, D., FUCHS, W., HOFFMANN, B., GRANZOW, H., STARICK, E., MUNDT, E., SCHIRRMEIER, H., MEBATSION, T. & METTENLEITER, T. C. 2006. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. *Proceedings of the National Academy of Sciences,* 103**,** 8197-8202.

VERHAGEN, J. H., FOUCHIER, R. A. & LEWIS, N. 2021. Highly pathogenic avian influenza viruses at the wild–domestic bird interface in europe: Future directions for research and surveillance. *Viruses,* 13**,** 212.

VEY, M., ORLICH, M., ADLER, S., KLENK, H.-D., ROTT, R. & GARTEN, W. 1992. Hemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif RXK/RR. *Virology,* 188**,** 408-413.

VIRALZONE. 2010. *Structure of Influenza virus [Online]* [Online]. Swiss Institute of bioinformatics. Available: https://viralzone.expasy.org/223 [Accessed 12 December 2019].

WADEE, A., KUSCHKE, R. & DOOMS, T. 1995. The inhibitory effects of Mycobacterium tuberculosis on MHC class II expression by monocytes activated with riminophenazines and phagocyte stimulants. *Clinical & Experimental Immunology,* 100**,** 434-439.

WAGNER, R., MATROSOVICH, M. & KLENK, H. D. 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev Med Virol,* 12**,** 159-66.

WAN, H. & PEREZ, D. R. 2006. Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. *Virology,* 346**,** 278-286.

WAN, H., SORRELL, E. M., SONG, H., HOSSAIN, M. J., RAMIREZ-NIETO, G., MONNE, I., STEVENS, J., CATTOLI, G., CAPUA, I., CHEN, L. M., DONIS, R. O., BUSCH, J., PAULSON, J. C., BROCKWELL, C., WEBBY, R., BLANCO, J., AL-NATOUR, M. Q. & PEREZ, D. R. 2008. Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. *PLoS One,* 3**,** e2923.

WANG, P., PALESE, P. & O'NEILL, R. E. 1997. The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal. *Journal of virology,* 71**,** 1850-1856.

WANG, Q., MEI, C., ZHEN, H. & ZHU, J. 2012. Codon preference optimization increases prokaryotic cystatin C expression. *Journal of Biomedicine and Biotechnology,* 2012.

WANG, R. & TAUBENBERGER, J. K. 2010. Methods for molecular surveillance of influenza. *Expert review of anti-infective therapy,* 8**,** 517-527.

WANG, S. H., CHEN, J., SMITH, D., CAO, Z., ACOSTA, H., FAN, Y., CIOTTI, S., FATTOM, A. & BAKER JR, J. 2020. A novel combination of intramuscular vaccine adjuvants, nanoemulsion and CpG produces an effective immune response against influenza A virus. *Vaccine,* 38**,** 3537-3544.

WANG, S. H., SMITH, D., CAO, Z., CHEN, J., ACOSTA, H., CHICHESTER, J. A., YUSIBOV, V., STREATFIELD, S. J., FATTOM, A. & BAKER JR, J. R. 2019. Recombinant H5 hemagglutinin adjuvanted with nanoemulsion protects ferrets against pathogenic avian influenza virus challenge. *Vaccine,* 37**,** 1591-1600.

WANG, W., BUTLER, E. N., VEGUILLA, V., VASSELL, R., THOMAS, J. T., MOOS JR, M., YE, Z., HANCOCK, K. & WEISS, C. D. 2008. Establishment of retroviral pseudotypes with influenza hemagglutinins from H1, H3, and H5 subtypes for sensitive and specific detection of neutralizing antibodies. *Journal of virological methods,* 153**,** 111-119.

WANG, W. & KRUG, R. M. 1998. U6atac snRNA, the highly divergent counterpart of U6 snRNA, is the specific target that mediates inhibition of AT-AC splicing by the influenza virus NS1 protein. *Rna* 4**,** 55-64.

WANG, W., LU, B., ZHOU, H., SUGUITAN, A. L., CHENG, X., SUBBARAO, K., KEMBLE, G. & JIN, H. 2010. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. *Journal of virology,* 84**,** 6570-6577.

WARD, B. J., MAKARKOV, A., SÉGUIN, A., PILLET, S., TRÉPANIER, S., DHALIWALL, J., LIBMAN, M. D., VESIKARI, T. & LANDRY, N. 2020. Efficacy, immunogenicity, and safety of a plant-derived, quadrivalent, virus-like particle influenza vaccine in adults (18–64 years) and older adults (≥ 65 years): two multicentre, randomised phase 3 trials. *The Lancet,* 396**,** 1491-1503.

WARD, C. W. & DOPHEIDE, T. A. 1981. Amino acid sequence and oligosaccharide distribution of the haemagglutinin from an early Hong Kong influenza virus variant A/Aichi/2/68 (X-31). *Biochem J,* 193**,** 953-62.

WEBBY, R. J. A. W., E.A. 2015. Centralized consensus hemagglutinin genes induce protective immunity against H1, H3 and H5 influenza viruses. *PloS one,* 10.

WEBER, T. P. & STILIANAKIS, N. I. 2008. Inactivation of influenza A viruses in the environment and modes of transmission: a critical review. *Journal of infection,* 57**,** 361-373.

WEBSTER, G. R., TEH, A. Y. H. & MA, J. K. C. 2017. Synthetic gene design—The rationale for codon optimization and implications for molecular pharming in plants. *Biotechnology and bioengineering,* 114**,** 492-502.

WEBSTER, R. G., BEAN, W. J., GORMAN, O. T., CHAMBERS, T. M. & KAWAOKA, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiological reviews,* 56**,** 152-179.

WEBSTER, R. G. & GOVORKOVA, E. A. 2014. Continuing challenges in influenza. *Annals of the New York Academy of Sciences,* 1323**,** 115-139.

WEBSTER, R. G. & ROTT, R. 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. *Cell,* 50**,** 665-6.

WEIS, W., BROWN, J., CUSACK, S., PAULSON, J., SKEHEL, J. & WILEY, D. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature,* 333**,** 426-431.

WHITE, J., HELENIUS, A. & GETHING, M.-J. 1982. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. *Nature,* 300**,** 658-659.

WHITE, J., MATLIN, K. & HELENIUS, A. 1981. Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. *The Journal of cell biology,* 89**,** 674-679.

WHITE, J. M. & WHITTAKER, G. R. 2016. Fusion of enveloped viruses in endosomes. *Traffic,* 17**,** 593-614.

WHITMIRE, J. K., TAN, J. T. & WHITTON, J. L. 2005. Interferon-γ acts directly on CD8+ T cells to increase their abundance during virus infection. *The Journal of experimental medicine,* 201**,** 1053-1059.

WHITTAKER, G., KEMLER, I. & HELENIUS, A. 1995. Hyperphosphorylation of mutant influenza virus matrix protein, M1, causes its retention in the nucleus. *J Virol,* 69**,** 439-45.

WHO. 2016. *WHO, 2016, Influenza (seasonal) fact sheet (online)* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs211/en/ [Accessed November 11, 2019].

WHO. 2017. *Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO.* [Online]. World Health Organisation. Available: https://www.who.int/influenza/human_animal_interface/2018_03_02_tableH5N1.pdf [Accessed Mrach 31, 2020].

WHO. 2022. *Influenza A(H5N1) Vaccine Stockpile and Inter-Pandemic Vaccine Use* [Online]. Available: https://www.who.int/immunization/sage/meetings/2013/november/SAGE_WG_H5vaccine_background_paper_16Oct2013_v4.pdf [Accessed March 22, 2020].

WIBAWA, H., BINGHAM, J., NURADJI, H., LOWTHER, S., PAYNE, J., HARPER, J., WONG, F., LUNT, R., JUNAIDI, A., MIDDLETON, D. & MEERS, J. 2013. The pathobiology of two Indonesian H5N1 avian influenza viruses representing different clade 2.1 sublineages in chickens and ducks. *Comp Immunol Microbiol Infect Dis,* 36**,** 175-91.

WILLE, M. & BARR, I. G. 2022. Resurgence of avian influenza virus. *Science,* 376**,** 459-460.

WILLIAMS, C. & ZEDEK, A. 2010. Comparative field evaluations of in ovo applied technology. *Poultry science,* 89**,** 189-193.

WILSON, I. A., SKEHEL, J. J. & WILEY, D. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature,* 289**,** 366-373.

WINTER, G. & FIELDS, S. 1982. Nucteotide sequence of human influenza A/PR/8/34 segment 2. *Nucleic Acids Research,* 10**,** 2135-2143.

WISE, H. M., FOEGLEIN, A., SUN, J., DALTON, R. M., PATEL, S., HOWARD, W., ANDERSON, E. C., BARCLAY, W. S. & DIGARD, P. 2009. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *Journal of virology,* 83**,** 8021-8031.

WISE, H. M., HUTCHINSON, E. C., JAGGER, B. W., STUART, A. D., KANG, Z. H., ROBB, N., SCHWARTZMAN, L. M., KASH, J. C., FODOR, E. & FIRTH, A. E. 2012. Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. *PLoS pathogens,* 8**,** e1002998.

WOAH. 2021. *Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 3.3.4. Avian influenza (Infection with avian influenza viruses)* [Online]. Available: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf [Accessed 7 January 2023].

WRIGHT, P. F., NEUMANN, G. AND KAWAOKA, Y. 2007. *Fields Virology*.

WROBLEWSKI, T., TOMCZAK, A. & MICHELMORE, R. 2005. Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. *Plant Biotechnology Journal,* 3**,** 259-273.

WYDRO, M., KOZUBEK, E. & LEHMANN, P. 2006. Optimization of transient Agrobacterium-mediated gene expression system in leaves of Nicotiana benthamiana. *Acta biochimica polonica,* 53**,** 289-298.

XIE, R., EDWARDS, K. M., WILLE, M., WEI, X., WONG, S.-S., ZANIN, M., EL-SHESHENY, R., DUCATEZ, M., POON, L. L. & KAYALI, G. 2022. The episodic resurgence of highly pathogenic avian influenza H5 virus. *bioRxiv*.

XU, X., SUBBARAO, K., COX, N. J. & GUO, Y. 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology,* 261**,** 15-19.

YÁNGÜEZ, E., CASTELLO, A., WELNOWSKA, E., CARRASCO, L., GOODFELLOW, I. & NIETO, A. 2011. Functional impairment of eIF4A and eIF4G factors correlates with inhibition of influenza virus mRNA translation. *Virology,* 413**,** 93-102.

YÁNGÜEZ, E., RODRIGUEZ, P., GOODFELLOW, I. & NIETO, A. 2012. Influenza virus polymerase confers independence of the cellular cap-binding factor eIF4E for viral mRNA translation. *Virology,* 422**,** 297-307.

YASUDA, J., NAKADA, S., KATO, A., TOYODA, T. & ISHIHAMA, A. 1993. Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. *Virology,* 196**,** 249-55.

YE, Z., LIU, T., OFFRINGA, D. P., MCINNIS, J. & LEVANDOWSKI, R. A. 1999. Association of influenza virus matrix protein with ribonucleoproteins. *J Virol,* 73**,** 7467-73.

YE, Z., ROBINSON, D. & WAGNER, R. R. 1995. Nucleus-targeting domain of the matrix protein (M1) of influenza virus. *Journal of Virology,* 69**,** 1964-1970.

YEWDELL, J. W., BENNINK, J. R., SMITH, G. L. & MOSS, B. 1985. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A,* 82**,** 1785-9.

YORK, I. A., STEVENS, J. & ALYMOVA, I. V. 2019. Influenza virus N-linked glycosylation and innate immunity. *Bioscience Reports,* 39.

YOSHIMURA, A. & OHNISHI, S. 1984. Uncoating of influenza virus in endosomes. *Journal of Virology,* 51**,** 497-504.

ZAMBON, M. C. 1999. Epidemiology and pathogenesis of influenza. *Journal of Antimicrobial Chemotherapy,* 44**,** 3-9.

ZAMBON, M. C. 2001. The pathogenesis of influenza in humans. *Reviews in medical virology,* 11**,** 227-241.

ZECCHIN, B., MINOUNGOU, G., FUSARO, A., MOCTAR, S., OUEDRAOGO-KABORÉ, A., SCHIVO, A., SALVIATO, A., MARCIANO, S. & MONNE, I. 2017. Influenza A (H9N2) Virus, Burkina Faso. *Emerging infectious diseases,* 23**,** 2118.

ZENG, J., FOURNIER, P. & SCHIRRMACHER, V. 2002. Stimulation of human natural interferon-alpha response via paramyxovirus hemagglutinin lectin-cell interaction. *J Mol Med (Berl),* 80**,** 443-51.

ZHANG, J. & LAMB, R. A. 1996. Characterization of the membrane association of the influenza virus matrix protein in living cells. *Virology,* 225**,** 255-66.

ZHANG, J., MA, K., LI, B., CHEN, Y., QIU, Z., XING, J., HUANG, J., HU, C., HUANG, Y. & LI, H. 2021. A risk marker of tribasic hemagglutinin cleavage site in influenza A (H9N2) virus. *Communications biology,* 4**,** 1-13.

ZHANG, J., PEKOSZ, A. & LAMB, R. A. 2000. Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. *J Virol,* 74**,** 4634-44.

ZHAO, M., VANDERSLUIS, M., STOUT, J., HAUPTS, U., SANDERS, M. & JACQUEMART, R. 2019. Affinity chromatography for vaccines manufacturing: Finally ready for prime time? *Vaccine,* 37**,** 5491-5503.

ZHOU, S. & STANDRING, D. N. 1992. Hepatitis B virus capsid particles are assembled from core-protein dimer precursors. *Proceedings of the National Academy of Sciences,* 89**,** 10046-10050.

ZHOU, X.-F., ZHANG, C.-L., GAO, X.-P., WANG, W.-L., HE, Z.-F., JIANG, F.-Y., PANG, Y.-L., LI, J.-H., REN, X.-J., ZHOU, H.-B., TAN, G.-Q., LYU, J.-X. & WANG, W. 2020. A simple and rapid protein purification method based on cell-surface display of SUMO-fused recombinant protein and Ulp1 protease. *AMB Express,* 10**,** 65.

ZUO, X., LI, S., HALL, J., MATTERN, M. R., TRAN, H., SHOO, J., TAN, R., WEISS, S. R. & BUTT, T. R. 2005. Enhanced expression and purification of membrane proteins by SUMO fusion in Escherichia coli. *J Struct Funct Genomics,* 6**,** 103-11.

Appendix A: List of Haemagglutinin (H5) present in chickens in the world.

Accession number	H5 subtype	Country	Year Isolated	Nucleotide length (bp)
1- MK411208	H5N6	Bangladesh	2017	1776
2- MH998391	H5N8	Egypt	2017	1742
3- MT256068	H5N8	Egypt	2019	1704
4- MF166575	H5N8	Israel	2016	1717
5- MK922520	H5N8	Saudi Arabia	2018	1704
6- MG650619	H5N8	Cameroon	2017	1704
7- MH998472	H5N8	Egypt	2017	1742
8- MH569621	H5N8	Uganda	2017	1704
9- MH165820	H5N8	South Africa	2017	1704
10- MF973225	H5N8	Zimbabwe	2017	1704
11- MN252530	H5N8	South Africa	2018	1704
12- MT027086	H5N8	Slovakia	2020	1704
13- MF425636	H5N8	Belgium	2017	1704

14-MH986133	H5N8	Egypt	2018	1717
15-MF926453	H5N8	Russia	2017	1742
16-MN038195	H5N8	Egypt	2018	1704
17-MN038194	H5N8	Egypt	2018	1704
18-MF073901	H5N8	Belgium	2017	1739
19-KY621534	H5N8	Czech Republic	2017	1742
20-MH998470	H5N8	Egypt	2017	1742
21-KP739405	H5N8	Oregon	2014	1744
22-KU646909	H5N3	Taiwan	2015	1776
23-KU201792	H5N8	California	2015	1744
24-KP795737	H5N2	Canada	2014	1704

25-MN988805	H5N2	Taiwan	2019	1776
26-AB932556	H5N8	Japan	2014	1742
27- KJ509044	H5N8	Korea	2014	1742
28- KJ509124	H5N8	Korea	2014	1742
29-KY415626	H5N6	China	2016	1743
30-LC208492	H5N6	Japan	2015	1766
31-MT547570	H5N1	Vietnam	2019	1758
32-LC376799	H5N6	Vietnam	2017	1704
33-KP286109	H5N6	Vietnam	2013	1704
34-KP090439	H5N6	China	2014	1776
35-KY171732	H5N6	Vietnam	2015	1704
36-KU143267	H5N6	China	2015	1771

37-KP735814	H5N1	China	2011	1704
38-CY095701	H5N1	Vietnam	2009	1776
39-CY099968	H5N1	Vietnam	2008	1776
40-GU052486	H5N1	China	2006	1751
41- JX420174	H5N1	Turkey	2007	1704
42-JQ936687	H5N1	Myanmar	2010	1707
43-GQ412056	H5N1	Korea	2008	1700
44- FJ654295	H5N1	Russia	2008	1757
45-KF169907	H5N1	China	2009	1707
46-LC208484	H5N1	Japan	2015	1766
47-KY776496	H5N1	China	2015	1704
48-KM110971	H5N1	India	2013	1732
49-KP861974	H5N1	Nepal	2013	1732
50-KM386913	H5N1	India	2011	1748

51-KM110976	H5N1	India	2012	1732
52-KF888461	H5N1	Bangladesh	2012	1704
53-MG042464	H5N1	Bangladesh	2016	1751
54-KM267884	H5N1	Bangladesh	2014	1704
55-KY635800	H5N1	Bangladesh	2015	1731
56-MN994193	H5N1	Bangladesh	2017	1704
57-KT285357	H5N1	India	2015	1739
58-KR732552	H5N1	Vietnam	2010	1776
59-JN807982	H5N1	Korea	2011	1704
60-JN613388	H5N1	Nepal	2010	1702
61-KJ522766	H5N1	China	2010	1704
62-KY614991	H5N1	Indonesia	2016	1776
63-MN046350	H5N1	Myanmar	2017	1704
64-KY614832	H5N1	Indonesia	2014	1771
65-KP762501	H5N1	China	2013	1742
66-KY171350	H5N1	Vietnam	2012	1704

67-MH285298	H5N1	Cambodia	2015	1704
68-LC194831	H5N1	Vietnam	2015	1726
69-KX644141	H5N1	Lebanon	2016	1778
70-KY926771	H5N1	Cameroon	2016	1704
71-MF112632	H5N1	Nigeria	2016	1704
72-KU971341	H5N1	Niger	2015	1704
73-MF112617	H5N1	Nigeria	2015	1704
74-KU971469	H5N1	Ghana	2015	1704
75-KU971554	H5N1	Burkina Faso	2015	1704
76-KU971570	H5N1	Ivory Coast	2015	1704
77-JF827081	H5N1	Pakistan	2008	1796
78-CY068910	H5N1	Pakistan	2010	1707
79-CY043367	H5N1	Bangladesh	2008	1679
80-GU354081	H5N1	India	2009	1762
81-JN679058	H5N1	Bangladesh	2007	1676
82-CY090116	H5N1	India	2010	1735
83-KT327366	H5N1	India	2008	1707
84-KF888416	H5N1	Bangladesh	2010	1688
85-CY043374	H5N1	Bangladesh	2009	1708
86-CY105894	H5N1	Ukraine	2008	1735

87-FM163440	H5N1	Poland	2007	1707
88-EU332353	H5N1	Romania	2007	1682
89-FM177119	H5N1	Germany	2007	1779
90-EU443553	H5N1	Czech Republic	2007	1745
91-EU233699	H5N1	Korea	2006	1738
92-CY021373	H5N1	Afghanistan	2006	1731
93-EU373734	H5N1	Ghana	2007	1707
94-EF474450	H5N1	Russia	2007	1754
95-FJ445017	H5N1	Iran	2008	1708
96-DQ887062	H5N1	India	2006	1723
97-DQ838517	H5N1	Niger	2006	1707
98-CY020677	H5N1	Sudan	2006	1744
99-EU148404	H5N1	Nigeria	2007	1718
100-AM262542	H5N1	Nigeria	2006	1727
101-DQ676834	H5N1	Russia	2006	1754

102-HQ724523	H5N1	Russia	2005	1758
103-FJ785142	H5N1	Romania	2006	1668
104-HQ198252	H5N1	Egypt	2009	1710
105-HM466695	H5N1	Israel	2010	1751
106-HQ198296	H5N1	Egypt	2010	1707
107-EU574927	H5N1	Israel	2008	1715
108-EU496384	H5N1	Egypt	2006	1736
109-EF532630	H5N1	Palestine	2006	1721
110-EF532629	H5N1	Israel	2006	1721
111-GQ184249	H5N1	Egypt	2008	1707
112-HQ198254	H5N1	Egypt	2009	1707

113-EU183332	H5N1	Egypt	2007	1704
114-KT220544	H5N1	Egypt	2013	1747
115-CY099588	H5N1	Egypt	2011	1742
116-KF258176	H5N1	Egypt	2012	1742
117-KP209302	H5N1	Egypt	2014	1704
118-JN807775	H5N1	Egypt	2010	1741
119-MH998488	H5N1	Egypt	2017	1742
120-KY558850	H5N1	Egypt	2016	1742
121-MK190719	H5N1	Egypt	2015	1742
122-KR063678	H5N1	Egypt	2014	1710
123-KT792916	H5N1	Palestine	2015	1724

124-AB188824	H5N1	Japan	2004	1704
125-AB188816	H5N1	China	2004	1704
126-AY676035	H5N1	Korea	2003	1704
127-GQ122387	H5N1	Indonesia	2004	1707
128-EF473081	H5N1	Indonesia	2003	1748
129-MK963403	H5N2	Vietnam	2013	1707
130-KM892798	H5N1	Indonesia	2009	1779
131-AB621354	H5N1	Indonesia	2010	1751
132-CY091966	H5N1	Indonesia	2008	1746
133-CY091929	H5N1	Indonesia	2007	1746
134-KP742900	H5N1	Indonesia	2013	1707

135-KP742883	H5N1	Indonesia	2011	1707
136-EU547798	H5N1	Thailand	2008	1755
137-EU118132	H5N1	Vietnam	2004	1707
138-EU497921	H5N1	Thailand	2007	1736
139-HM590797	H5N1	Thailand	2010	1720
140-EF467802	H5N1	Thailand	2004	1720
141-KC684446	H5N1	Malaysia	2004	1707
142-JQ794462	H5N1	Thailand	2006	1736
143-EF566200	H5N1	Vietnam	2005	1707
144-HQ200562	H5N1	Cambodia	2005	1703
145-JN588944	H5N1	Cambodia	2008	1758

146-HQ200575	H5N1	Cambodia	2007	1770
147-JX420206	H5N1	Vietnam	2006	1779
148-JN588822	H5N1	Cambodia	2010	1764
149-KP097925	H5N1	Vietnam	2011	1707
150-JN588824	H5N1	Cambodia	2011	1759
151-KF369229	H5N1	Cambodia	2012	1707
152-KF918485	H5N1	Cambodia	2013	1775
153-KU311522	H5N1	Vietnam	2013	1710
154-DQ023145	H5N1	China	2002	1736
155-GU052135	H5N1	China	1997	1754
156-KF150634	H5N2	China	2013	1704

157-KY171509	H5N1	Vietnam	2013	1704
158-KF638579	H5N1	China	2012	1704
159-KU143255	H5N2	China	2015	1712
160-KU310447	H5N1	France	2015	1731
161-EF597263	H5N2	Italy	1997	1718
162-KM190930	H5N1	Scotland	1959	1692
163-KU143256	H5N2	China	2014	1752
164-MH546339	H5N2	New Jersey	2008	1734
165-KY063732	H5N1	New Jersey	2015	1695
166-GQ117129	H5N2	New York	2006	1742
167-GU049896	H5N2	Pennsylvania	2006	1607

168-CY034683	H5N3	Texas	2002	1767
169-GU052644	H5N2	Texas	2004	1742
170-GU052779	H5N2	Florida	1986	1742
171-KJ162708	H5N2	Taiwan	2013	1695
172-KY989967	H5N2	Taiwan	2012	1695
173-KY989969	H5N2	Taiwan	2010	1695
174-GU052690	H5N2	Mexico	1995	1742
175-KM455945	H5N2	Mexico	2006	1695
176-KM455897	H5N2	Mexico	2005	1695
177-GU052795	H5N2	Guatemala	2000	1742
178-EU099401	H5N2	Guatemala	2003	1742

Appendix B: List of Haemagglutinin (H9N2) present in chickens in the world.

Accession number	**Country**	**Year Isolated**	**Nucleotide length (bp)**
1-MG210366	Pakistan	2016	1683
2-MH105306	Pakistan	2018	1691
3-MG720815	Pakistan	2015	1683
4-MK159013	Iran	2017	1683
5-MH879789	Iraq	2016	1724
6-MH180388	Pakistan	2014	1683
7-KJ696534	Iran	2014	1733
8-JQ905259	Pakistan	2010	1714
9-CY038466	Pakistan	2008	1709
10-JX310067	India	2010	1683
11-JX273551	Nepal	2011	1741
12-KT285341	India	2012	1689
13-KT285333	India	2013	1694
14-MN242766	Kenya	2017	1715
15-MG881999	Uganda	2017	1727
16-JX273558	Saudi Arabia	2010	1741
17-JQ973658	Israel	2011	1680
18-MH558949	Israel	2017	1686

19-MF510852	Burkina Faso	2017	1683
20-MK240300	Algeria	2017	1707
21-KX351198	United Arab Emirates	2015	1683
22-JX273554	Pakistan	2006	1741
23-CY038482	Pakistan	2007	1741
24-KF188325	Pakistan	2005	1709
25-KJ696533	Iran	2013	1728
26-JQ419724	Iran	2009	1678
27-JN646748	Iran	2010	1738
28-JX456182	Iran	2011	1741
29-JQ973656	Israel	2012	1680
30-JQ254942	Israel	2010	1707
31-KF918709	Israel	2013	1680
32-FJ464729	Israel	2006	1707
33-KJ781213	Egypt	2013	1683
34-JQ440373	Egypt	2011	1739
35-KJ831058	Israel	2014	1680
36-KF258177	Egypt	2012	1707
37-KF881386	Egypt	2013	1693
38-KJ781216	Egypt	2013	1683
39-JQ906552	Egypt	2011	1715

40-JQ906551	Egypt	2012	1710
41-JQ906560	Egypt	2012	1691
42-CY126232	Egypt	2011	1696
43-KF258187	Egypt	2012	1707
44-KF881370	Egypt	2013	1704
45-KF998214	Egypt	2012	1683
46-LC534257	Egypt	2013	1683
47-KX000775	Egypt	2015	1741
48-KX000708	Egypt	2015	1715
49-KX783420	Israel	2016	1680
50-KY910830	Egypt	2016	1683
51-KX000838	Egypt	2015	1708
52-MN038193	Egypt	2018	1723
53-MK942499	Israel	2018	1686
54-MT499385	Israel	2019	1686
55-JX273556	Saudi Arabia	2006	1741
56-JX273560	Saudi Arabia	2006	1741
57-MG882009	Lebanon	2017	1718
58-JQ254937	Israel	2009	1709
59-GQ120561	Israel	2007	1744
60-JQ973661	Israel	2008	1680
61-JF323006	Tunisia	2010	1683

62-JX273540	Bangladesh	2007	1741
63-KM922684	India	2008	1741
64-MH045822	India	2009	1684
65-KC757824	Bangladesh	2011	1687
66-JX273545	Kuwait	2004	1741
67-KT362038	Bangladesh	2014	1683
68-MN209709	Bangladesh	2018	1716
69-MN994100	Bangladesh	2017	1683
70-MG042407	Bangladesh	2016	1716
71-KJ643856	Bangladesh	2012	1683
72-KC757802	Bangladesh	2010	1687
73-MT090569	Bangladesh	2019	1716
74-KJ643808	Bangladesh	2013	1683
75-KY635737	Bangladesh	2015	1716
76-GU071984	Iran	2007	1732
77-EF063731	Iran	2004	1683
78-GQ497133	Iran	2006	1680
79-EF063734	Iran	2005	1683
80-JX456177	Iran	2008	1741
81-KF188299	Pakistan	1999	1683
82-KF188352	United Arab Emirates	2001	1683

83-CY076723	United Arab Emirates	2002	1743
84-GU050554	Saudi Arabia	2002	1712
85-MF673311	Jordan	2004	1707
86-JX273546	Lebanon	2004	1742
87-JX273544	Israel	2004	1742
88-EF492240	Israel	2003	1707
89-MF673335	Jordan	2005	1707
90-MF673351	Jordan	2003	2003
91-GQ120553	Israel	2007	1744
92-EF492236	Israel	2005	1707
93-EF492231	Israel	2001	1707
94-EF492221	Israel	2000	1707
95-GQ497117	Iran	1998	1738
96-JX465626	Iran	1998	1738
97-CY081264	Saudi Arabia	1998	1680
98-EU477246	Iran	2002	1743
99-GQ497130	Iran	2004	1680
100-GQ497127	Iran	2003	1680
101-EF063727	Iran	2003	1683
102-GQ497132	Iran	2005	1669
103-EU477247	Iran	1999	1742

104-EU477248	Iran	2000	1742
105-GQ497120	Iran	1999	1681
106-GQ497123	Iran	2001	1683
107-GQ497121	Iran	2000	1681
108-DQ485208	China	2000	1742
109-AB256746	Japan	2001	1683
110-AB256714	Japan	2002	1683
111-JF906206	China	2010	1743
112-KC767259	China	2012	1742
113-MN918143	China	2019	1790
114-MN103741	China	2017	1742
115- MN103729	China	2016	1742
116- MN103716	China	2015	1742
117- LC208476	Japan	2015	1728
118- MN176651	Vietnam	2017	1730
119- MN103638	China	2013	1742
120- MH285290	Cambodia	2015	1683
121- MK964544	Vietnam	2013	1683
122- MK964275	Vietnam	2004	1717
123- MH560067	Vietnam	2013	1742
124- MN064851	China	2018	1683
125- MK979288	China	2014	1683

126- MN686275	China	2011	1683
127- MK979360	China	2012	1683
128- JX273541	Hungary	2001	1709
129- JX094859	Korea	2001	1742
130- KY785737	Korea	2002	1683
131- KY785729	Korea	2005	1711
132- EF620900	Korea	2000	1742
133- KF188387	Korea	1996	1689
134- KT157805	Korea	2008	1683
135- KF188395	Korea	2004	1689
136- MN530440	South Korea	2007	1683
137- AY790313	Korea	2003	1716
138- JN852803	Korea	2009	1683
139- MN530510	South Korea	2015	1683
140- MN530509	South Korea	2013	1683
141- JF917103	Korea	2010	1683
142- MN530492	South Korea	2011	1683
143- MN530499	South Korea	2012	1683

Appendix C: Equipment, apparatuses, and accessories.

Equipment	**Modell**	**Manufacturer**
Autoclave	Varioklav H + P 75 S(H+P)	Labortechnik, Oberschleißheim
Heating block	BTD	Grant Instrument, Cambridge
Gel electrophoresis	Power Pac Basic ™	BioRad, Segrate, Milan, Italy
Incubators	Thermo Fisher Scientific Stabilitherm Incubator	Thermo Scientific®
	Thermo Forma 420 Incubator Shaker	
UV trans–illuminator	Image Quant 300 imager	GE healthcare, UK
Spectrophotometer	NanoDrop 2000	Thermo Scientific®
Thermocycler	Gene Touch Plus Digital Gradient DNA PCR Machine Thermal Cycler	Hangzhou Bloer Technology, China
pH-Meter	Basic Meter PB-11	Sartorius AG, Göttingen
Balances	TE 6101	Sartorius, Göttingen
Water bath	Clifton NE1-14	Wolf Laboratories, Stafford, UK

Centrifuges	Heraeus™ Pico™ 17 Microcentrifuge	Thermo Scientific®
	Thermo Scientific Sorvall 75004373 Legend RT Plus	Thermo Scientific Sorvall, US
Vortex	Jencons PLS VX-100 Vortex Mixer	SciQuip, UK
Oven shaker	Hybridisation Oven, SI30H, Stuart	Stuart, UK
Ultra-pure water system	Sartorius™ H2O-II-1-UV-T	Thermo scientific, UK
electroporator	Eppendorf 2510	Eppendorf, UK
Centrifuge bottles	Nalgene™ PPCO Centrifuge Bottles with Sealing Closure	Thermo scientific, UK

Appendix D: Solutions, media, and buffers.

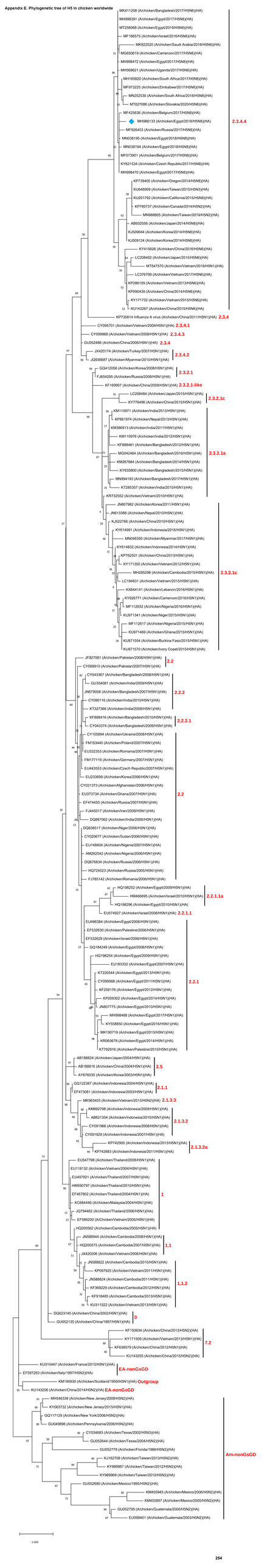
Buffer/Media/Supplement	**Reagent/pH/notes**
0.1 M Acetosyringone (10 ml)	0.196 g acetosyringone (Sigma-Aldrich),10 ml DMSO (dimethyl sulfoxide), prepare 1 ml aliquots, and store them at -20 °C

1 M MgCl2 (100 ml)	20.3 g MgCl2, complete with sterile deionized water, sterilize by autoclaving, and store at room temperature.
1 M MES (100 ml)	17.5 g MES, complete with sterile deionized water, adjust the pH to 5.6, sterilize by filtration, store at room temperature.
Lura Bertani Broth (LB-Broth)	10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0.
100 ml MMA	10 ml of 0.1 M MES pH 5.6, 1 ml of 1 M MgCl2, 100 ul of 0.1 M Acetosyringone, 88.9 ml H2O.
Lura Bertani Agar (LB-Agar)	10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 10 g/L Lab MN1 agar, pH 7.0.
1x TAE buffer	Tris base dissolved in 750mL of deionized water. The acetic acid was added and EDTA, then adjusted the volume to 1L by adding water. The pH of the 50x TAE buffer should be about 8.5. 49 parts of deionized water were

	added to 1 part of 50x TAE buffer to make the 1x TAE working buffer.
Kanamycin	50 mg/ml in water, stored at -20°C.
Ampicillin	100 mg/ml in water, stored at -20°C.
Rifampicin	10 mg/ml in methanol, stored at -20°C.
Old extraction buffer	50 mM of tris, 500 mM of NaCl, 10 mM of sodium thiosulphate, and 0.1%V/V Triton X-100
VLP extraction buffer	50 mM of tris, 75 mM of NaCl, and 0.1%V/V Triton X-100.
10% DMEM media	Dulbecco"s Modified Eagle Medium (Gibco® Thermo Scientific™, Altrincham, UK) supplemented with 1% L-glutamine (Gibco® Thermo Scientific™, Altrincham UK), 100 U penicillin, and streptomycin (1µg/ml) (Gibco® Thermo Scientific™, Altrincham, UK), and 10% fetal calf serum (Sigma, UK).

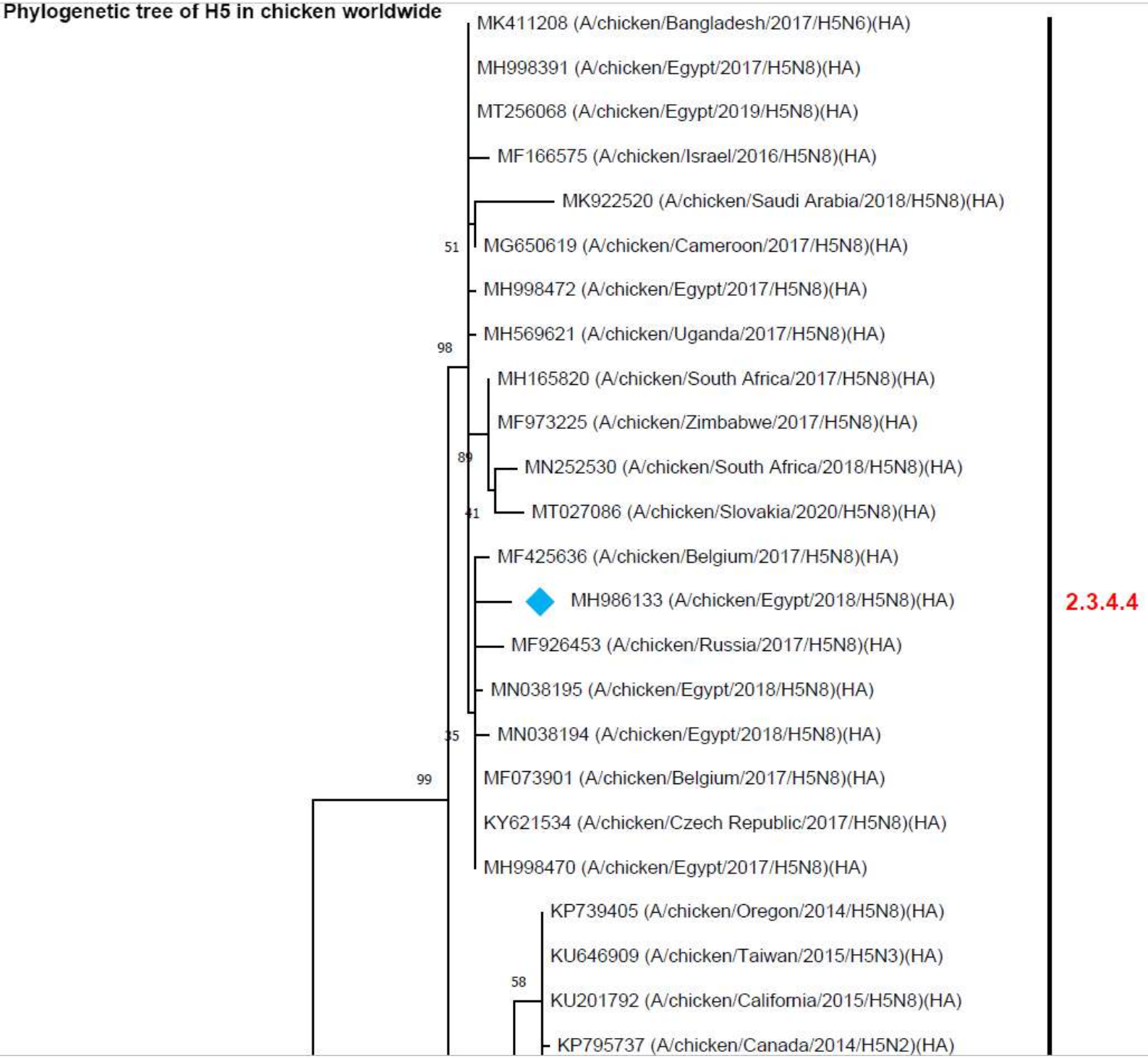
Prepared RPMI media	RPMI 1640 MediumThermo Scientific™,UK) supplemented with 1% L-glutamine (Gibco® Thermo Scientific™, Altrincham UK), 100 U penicillin, and streptomycin (1µg/ml) (Gibco® Thermo Scientific™, Altrincham, UK), 10% fetal calf serum (Sigma, UK),and 1% HEPES solution (Sigma, UK).
ELISA coating buffer	Carbonate-Bicarbonate buffer tablets (Sigma, Uk).
ELISA wash buffer	PBS-T (Posphate buffer saline supplemented with 20% tween (0.1%V/V).
ELISA blocking buffer	3% non-fat dry milk in PBS-T.

Appendix E. Phylogenetic tree of H5 in chicken worldwide

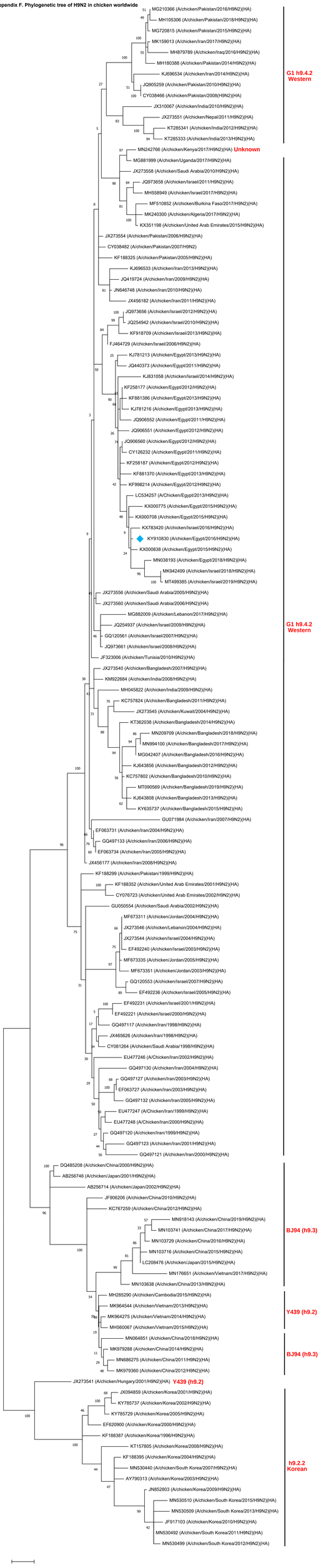


Appendix E

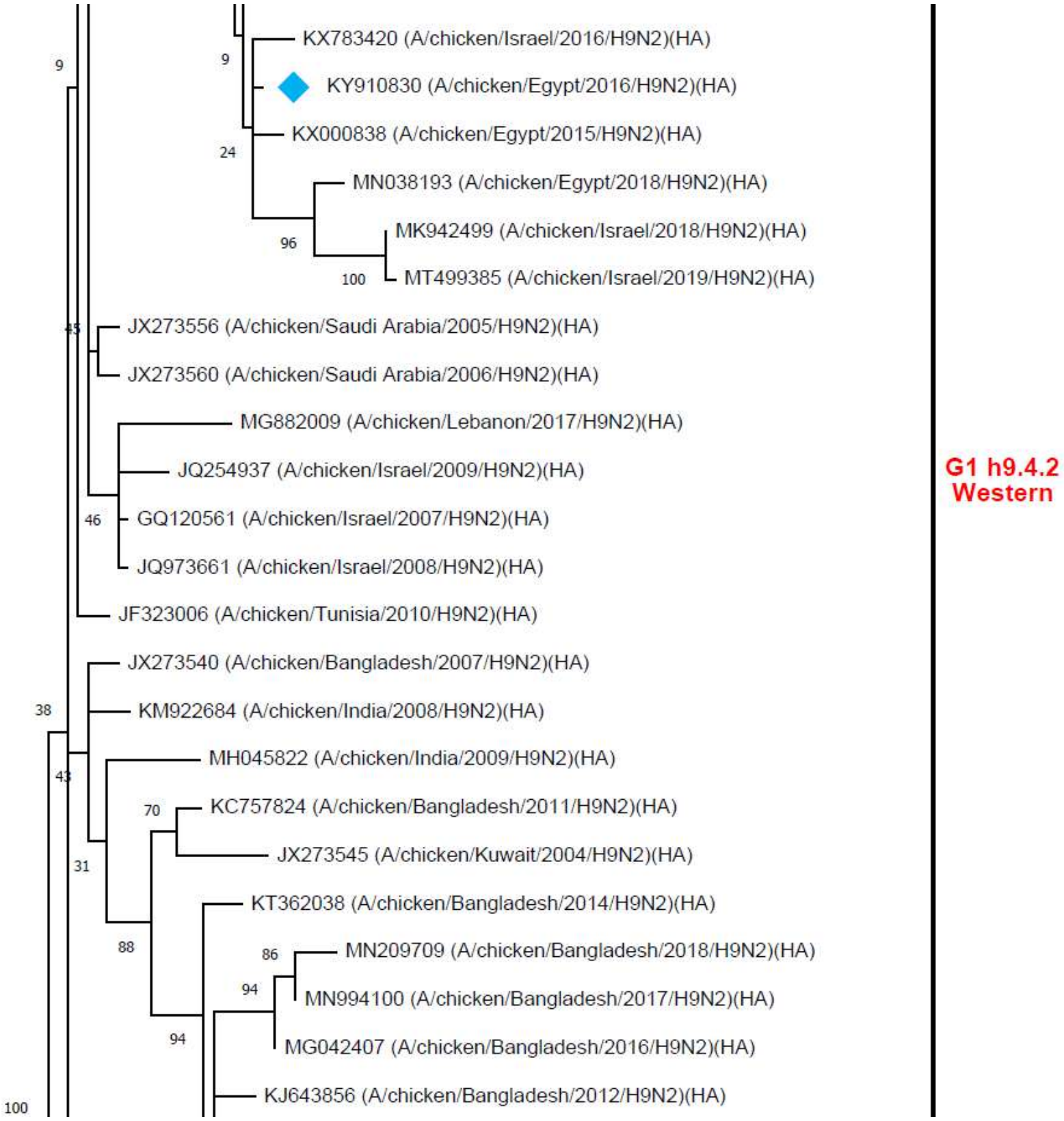
Phylogenetic tree of H5 in chicken worldwide



Appendix F. Phylogenetic tree of H9N2 in chicken worldwide

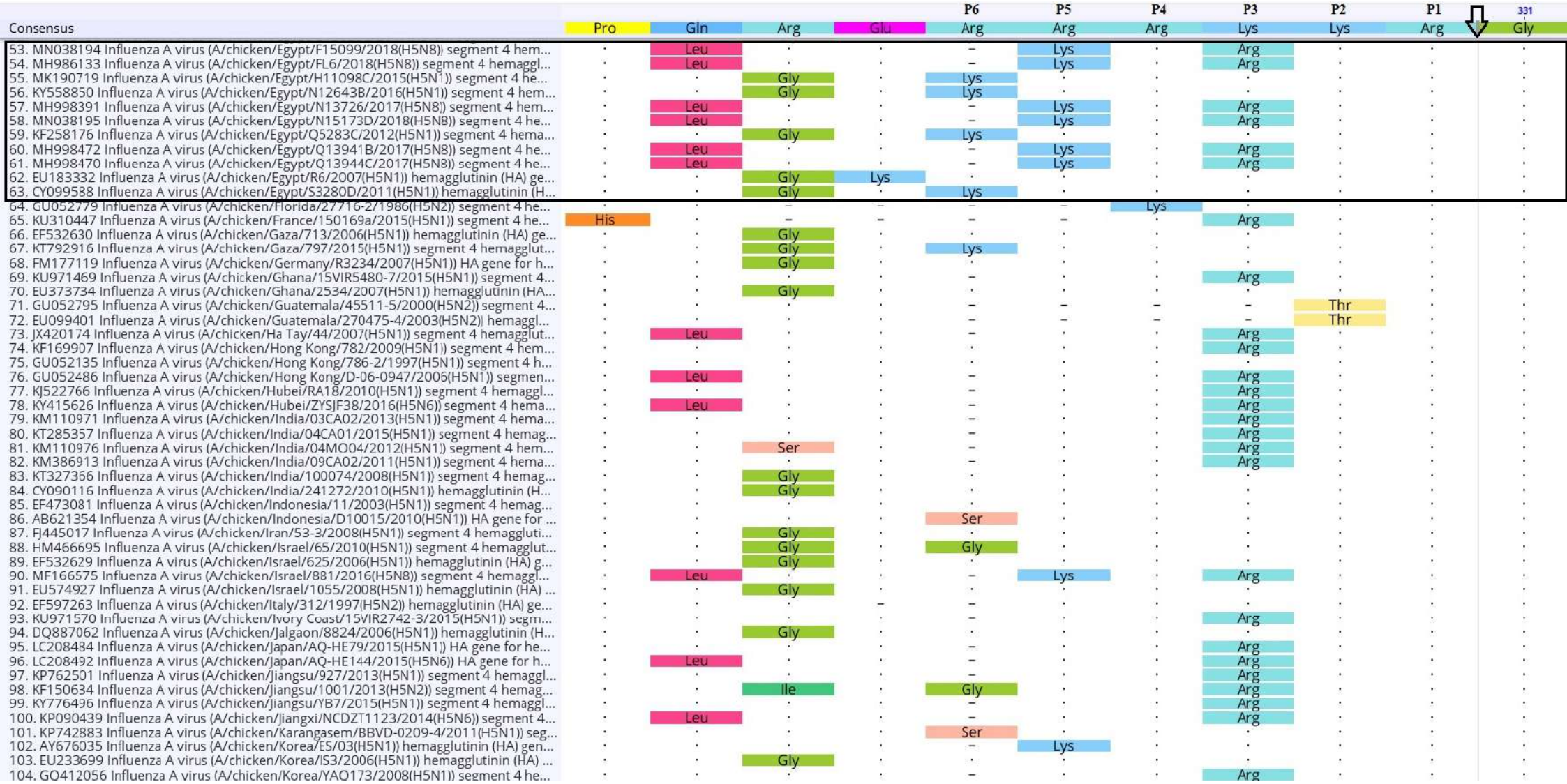


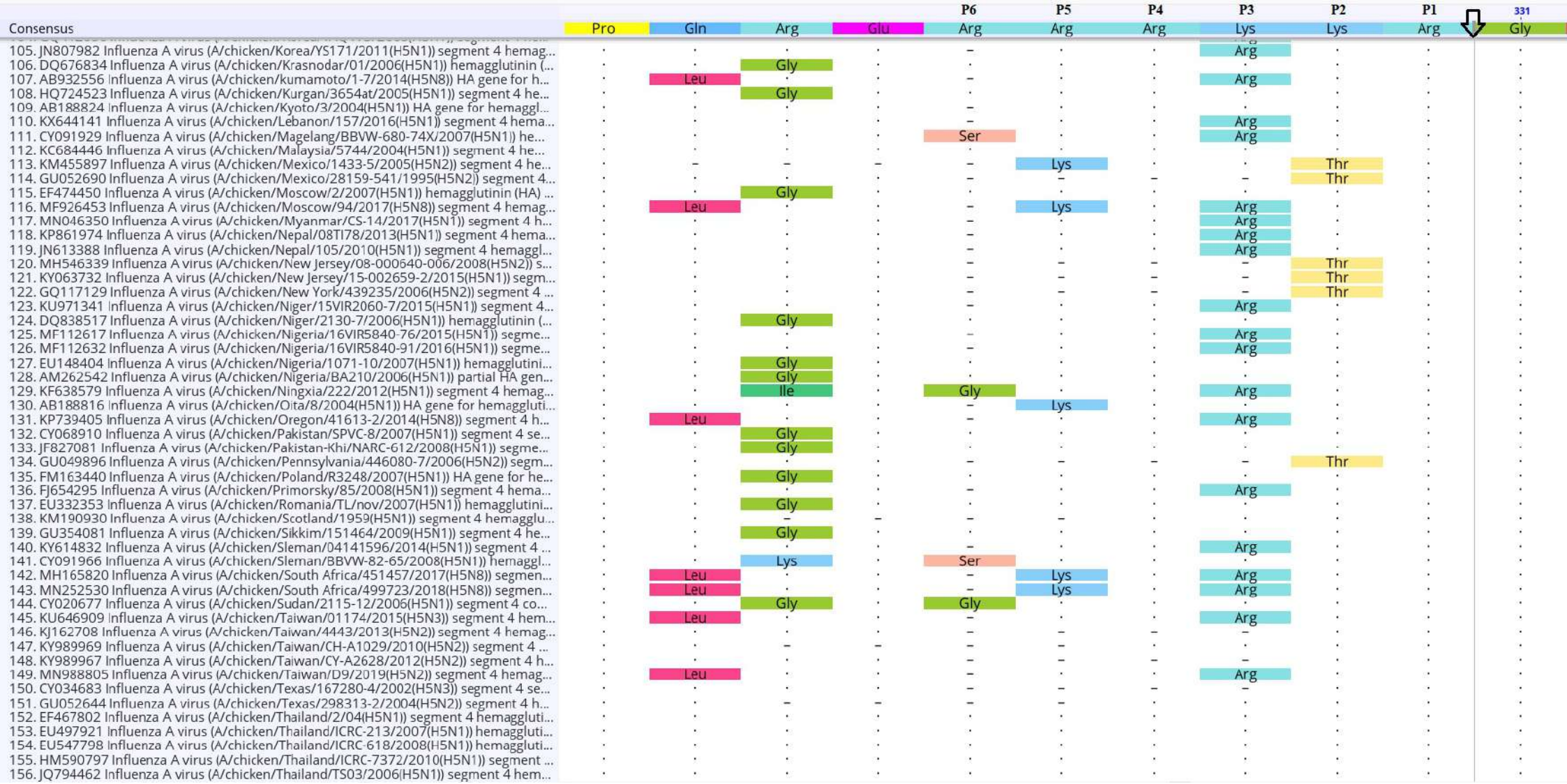
Appendix F. Phylogenetic tree of H9N2 in chicken worldwide

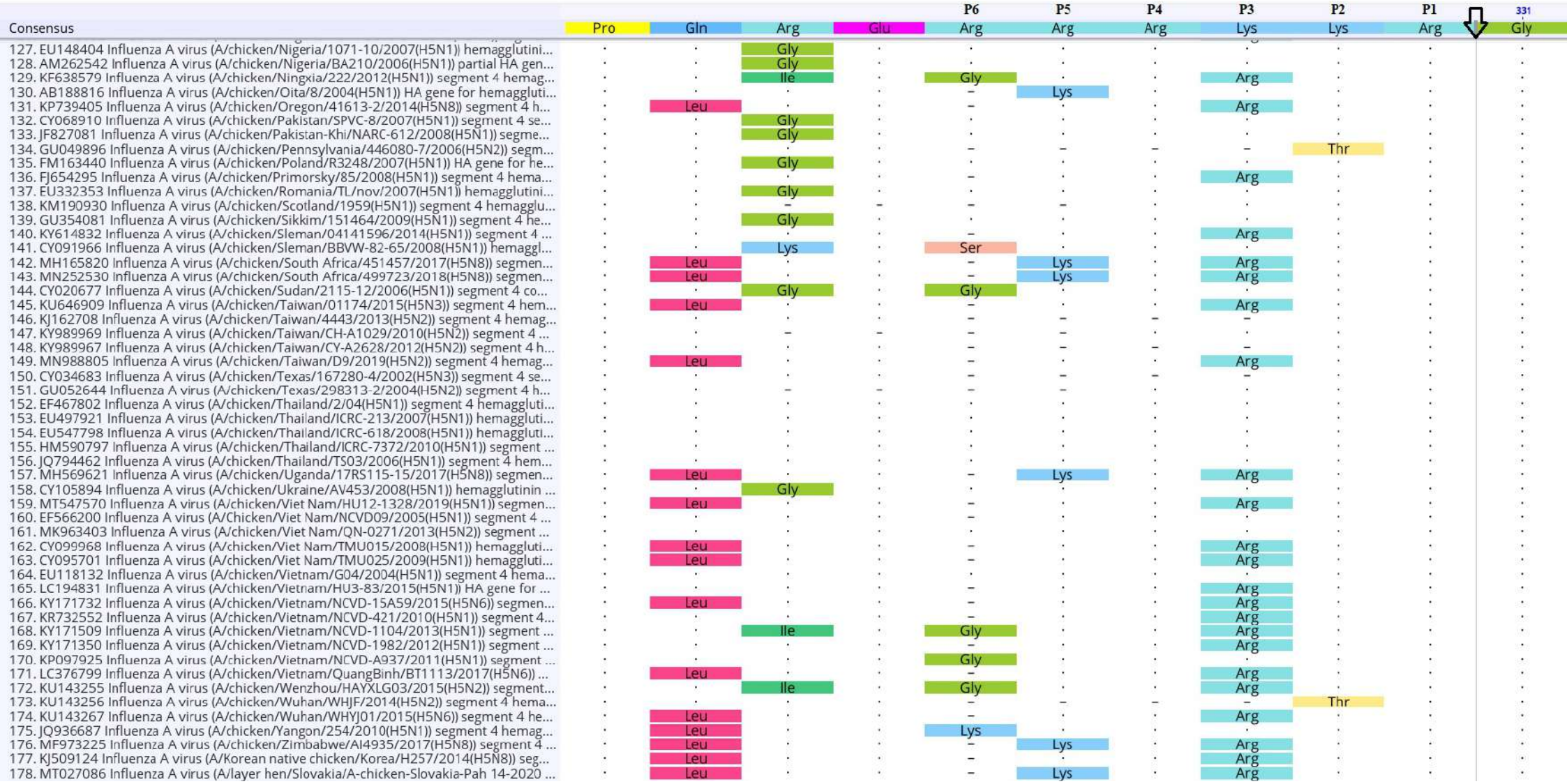


Appendix G. Cleavage site analysis of HPAIV H5 worldwide

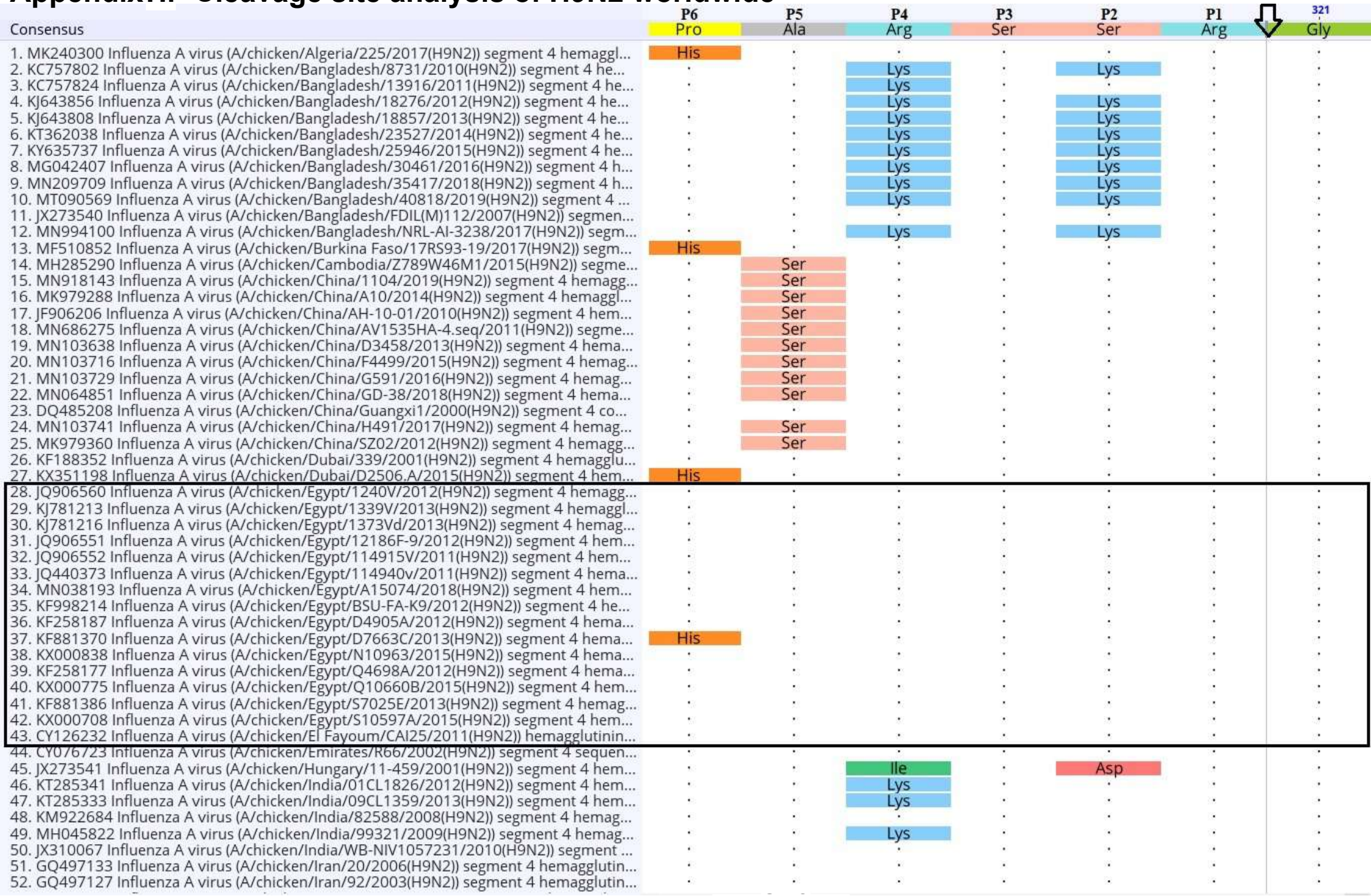



P6 P5 P4 P3 P2 P1 331
Consensus Pro Gln Arg Glu Arg Arg Arg Lys Lys Arg Gly
53. MN038194 Influenza A virus (A/chicken/Egypt/F15099/2018(H5N8)) segment 4 hem... Leu Lys Arg
54. MH986133 Influenza A virus (A/chicken/Egypt/FL6/2018(H5N8)) segment 4 hemaggl... Leu Lys Arg
55. MK190719 Influenza A virus (A/chicken/Egypt/H11098C/2015(H5N1)) segment 4 he... Gly Lys
56. KY558850 Influenza A virus (A/chicken/Egypt/N12643B/2016(H5N1)) segment 4 hem... Gly Lys
57. MH998391 Influenza A virus (A/chicken/Egypt/N13726/2017(H5N8)) segment 4 hem... Leu Lys Arg
58. MN038195 Influenza A virus (A/chicken/Egypt/N15173D/2018(H5N8)) segment 4 he... Leu Lys Arg
59. KF258176 Influenza A virus (A/chicken/Egypt/Q5283C/2012(H5N1)) segment 4 hema... Gly Lys
60. MH998472 Influenza A virus (A/chicken/Egypt/Q13941B/2017(H5N8)) segment 4 he... Leu Lys Arg
61. MH998470 Influenza A virus (A/chicken/Egypt/Q13944C/2017(H5N8)) segment 4 he... Leu Lys Arg
62. EU183332 Influenza A virus (A/chicken/Egypt/R6/2007(H5N1)) hemagglutinin (HA) ge... Gly Lys
63. CY099588 Influenza A virus (A/chicken/Egypt/S3280D/2011(H5N1)) hemagglutinin (H... Gly Lys
64. GU052779 Influenza A virus (A/chicken/Florida/27716-2/1986(H5N2)) segment 4 he... Lys
65. KU310447 Influenza A virus (A/chicken/France/150169a/2015(H5N1)) segment 4 he... His Arg
66. EF532630 Influenza A virus (A/chicken/Gaza/713/2006(H5N1)) hemagglutinin (HA) ge... Gly
67. KT792916 Influenza A virus (A/chicken/Gaza/797/2015(H5N1)) segment 4 hemagglut... Gly Lys
68. FM177119 Influenza A virus (A/chicken/Germany/R3234/2007(H5N1)) HA gene for h... Gly
69. KU971469 Influenza A virus (A/chicken/Ghana/15VIR5480-7/2015(H5N1)) segment 4... Arg
70. EU373734 Influenza A virus (A/chicken/Ghana/2534/2007(H5N1)) hemagglutinin (HA... Gly
71. GU052795 Influenza A virus (A/chicken/Guatemala/45511-5/2000(H5N2)) segment 4... Thr
72. EU099401 Influenza A virus (A/chicken/Guatemala/270475-4/2003(H5N2)) hemaggl... Thr
73. JX420174 Influenza A virus (A/chicken/Ha Tay/44/2007(H5N1)) segment 4 hemagglut... Leu Arg
74. KF169907 Influenza A virus (A/chicken/Hong Kong/782/2009(H5N1)) segment 4 hem... Arg
75. GU052135 Influenza A virus (A/chicken/Hong Kong/786-2/1997(H5N1)) segment 4 h...
76. GU052486 Influenza A virus (A/chicken/Hong Kong/D-06-0947/2006(H5N1)) segmen... Leu Arg
77. KJ522766 Influenza A virus (A/chicken/Hubei/RA18/2010(H5N1)) segment 4 hemaggl... Arg
78. KY415626 Influenza A virus (A/chicken/Hubei/ZYSJF38/2016(H5N6)) segment 4 hema... Leu Arg
79. KM110971 Influenza A virus (A/chicken/India/03CA02/2013(H5N1)) segment 4 hema... Arg
80. KT285357 Influenza A virus (A/chicken/India/04CA01/2015(H5N1)) segment 4 hemag... Arg
81. KM110976 Influenza A virus (A/chicken/India/04MO04/2012(H5N1)) segment 4 hem... Ser Arg
82. KM386913 Influenza A virus (A/chicken/India/09CA02/2011(H5N1)) segment 4 hema... Arg
83. KT327366 Influenza A virus (A/chicken/India/100074/2008(H5N1)) segment 4 hemag... Gly
84. CY090116 Influenza A virus (A/chicken/India/241272/2010(H5N1)) hemagglutinin (H... Gly
85. EF473081 Influenza A virus (A/chicken/Indonesia/11/2003(H5N1)) segment 4 hemag...
86. AB621354 Influenza A virus (A/chicken/Indonesia/D10015/2010(H5N1)) HA gene for ... Ser
87. FJ445017 Influenza A virus (A/chicken/Iran/53-3/2008(H5N1)) segment 4 hemaggluti... Gly
88. HM466695 Influenza A virus (A/chicken/Israel/65/2010(H5N1)) segment 4 hemagglut... Gly Gly
89. EF532629 Influenza A virus (A/chicken/Israel/625/2006(H5N1)) hemagglutinin (HA) g... Gly
90. MF166575 Influenza A virus (A/chicken/Israel/881/2016(H5N8)) segment 4 hemaggl... Leu Lys Arg
91. EU574927 Influenza A virus (A/chicken/Israel/1055/2008(H5N1)) hemagglutinin (HA) ... Gly
92. EF597263 Influenza A virus (A/chicken/Italy/312/1997(H5N2)) hemagglutinin (HA) ge...
93. KU971570 Influenza A virus (A/chicken/Ivory Coast/15VIR2742-3/2015(H5N1)) segm... Arg
94. DQ887062 Influenza A virus (A/chicken/Jalgaon/8824/2006(H5N1)) hemagglutinin (H... Gly
95. LC208484 Influenza A virus (A/chicken/Japan/AQ-HE79/2015(H5N1)) HA gene for he... Arg
96. LC208492 Influenza A virus (A/chicken/Japan/AQ-HE144/2015(H5N6)) HA gene for h... Leu Arg
97. KP762501 Influenza A virus (A/chicken/Jiangsu/927/2013(H5N1)) segment 4 hemaggl... Arg
98. KF150634 Influenza A virus (A/chicken/Jiangsu/1001/2013(H5N2)) segment 4 hemag... Ile Gly Arg
99. KY776496 Influenza A virus (A/chicken/Jiangsu/YB7/2015(H5N1)) segment 4 hemaggl... Arg
100. KP090439 Influenza A virus (A/chicken/Jiangxi/NCDZT1123/2014(H5N6)) segment 4... Leu Arg
101. KP742883 Influenza A virus (A/chicken/Karangasem/BBVD-0209-4/2011(H5N1)) seg... Ser
102. AY676035 Influenza A virus (A/chicken/Korea/ES/03(H5N1)) hemagglutinin (HA) gen... Lys
103. EU233699 Influenza A virus (A/chicken/Korea/IS3/2006(H5N1)) hemagglutinin (HA) ... Gly
104. GQ412056 Influenza A virus (A/chicken/Korea/YAQ173/2008(H5N1)) segment 4 he... Arg


Consensus
P6
P5
P4
P3
P2
P1
331
Pro Gln Arg Glu Arg Arg Arg Lys Lys Arg Gly
105. JN807982 Influenza A virus (A/chicken/Korea/YS171/2011(H5N1)) segment 4 hemag...
106. DQ676834 Influenza A virus (A/chicken/Krasnodar/01/2006(H5N1)) hemagglutinin (...
107. AB932556 Influenza A virus (A/chicken/kumamoto/1-7/2014(H5N8)) HA gene for h...
108. HQ724523 Influenza A virus (A/chicken/Kurgan/3654at/2005(H5N1)) segment 4 he...
109. AB188824 Influenza A virus (A/chicken/Kyoto/3/2004(H5N1)) HA gene for hemaggl...
110. KX644141 Influenza A virus (A/chicken/Lebanon/157/2016(H5N1)) segment 4 hema...
111. CY091929 Influenza A virus (A/chicken/Magelang/BBVW-680-74X/2007(H5N1)) he...
112. KC684446 Influenza A virus (A/chicken/Malaysia/5744/2004(H5N1)) segment 4 he...
113. KM455897 Influenza A virus (A/chicken/Mexico/1433-5/2005(H5N2)) segment 4 he...
114. GU052690 Influenza A virus (A/chicken/Mexico/28159-541/1995(H5N2)) segment 4...
115. EF474450 Influenza A virus (A/chicken/Moscow/2/2007(H5N1)) hemagglutinin (HA) ...
116. MF926453 Influenza A virus (A/chicken/Moscow/94/2017(H5N8)) segment 4 hemag...
117. MN046350 Influenza A virus (A/chicken/Myanmar/CS-14/2017(H5N1)) segment 4 h...
118. KP861974 Influenza A virus (A/chicken/Nepal/08TI78/2013(H5N1)) segment 4 hema...
119. JN613388 Influenza A virus (A/chicken/Nepal/105/2010(H5N1)) segment 4 hemaggl...
120. MH546339 Influenza A virus (A/chicken/New Jersey/08-000640-006/2008(H5N2)) s...
121. KY063732 Influenza A virus (A/chicken/New Jersey/15-002659-2/2015(H5N1)) segm...
122. GQ117129 Influenza A virus (A/chicken/New York/439235/2006(H5N2)) segment 4 ...
123. KU971341 Influenza A virus (A/chicken/Niger/15VIR2060-7/2015(H5N1)) segment 4...
124. DQ838517 Influenza A virus (A/chicken/Niger/2130-7/2006(H5N1)) hemagglutinin (...
125. MF112617 Influenza A virus (A/chicken/Nigeria/16VIR5840-76/2015(H5N1)) segme...
126. MF112632 Influenza A virus (A/chicken/Nigeria/16VIR5840-91/2016(H5N1)) segme...
127. EU148404 Influenza A virus (A/chicken/Nigeria/1071-10/2007(H5N1)) hemagglutini...
128. AM262542 Influenza A virus (A/chicken/Nigeria/BA210/2006(H5N1)) partial HA gen...
129. KF638579 Influenza A virus (A/chicken/Ningxia/222/2012(H5N1)) segment 4 hemag...
130. AB188816 Influenza A virus (A/chicken/Oita/8/2004(H5N1)) HA gene for hemaggluti...
131. KP739405 Influenza A virus (A/chicken/Oregon/41613-2/2014(H5N8)) segment 4 h...
132. CY068910 Influenza A virus (A/chicken/Pakistan/SPVC-8/2007(H5N1)) segment 4 se...
133. JF827081 Influenza A virus (A/chicken/Pakistan-Khi/NARC-612/2008(H5N1)) segme...
134. GU049896 Influenza A virus (A/chicken/Pennsylvania/446080-7/2006(H5N2)) segm...
135. FM163440 Influenza A virus (A/chicken/Poland/R3248/2007(H5N1)) HA gene for he...
136. FJ654295 Influenza A virus (A/chicken/Primorsky/85/2008(H5N1)) segment 4 hema...
137. EU332353 Influenza A virus (A/chicken/Romania/TL/nov/2007(H5N1)) hemagglutini...
138. KM190930 Influenza A virus (A/chicken/Scotland/1959(H5N1)) segment 4 hemagglu...
139. GU354081 Influenza A virus (A/chicken/Sikkim/151464/2009(H5N1)) segment 4 he...
140. KY614832 Influenza A virus (A/chicken/Sleman/04141596/2014(H5N1)) segment 4 ...
141. CY091966 Influenza A virus (A/chicken/Sleman/BBVW-82-65/2008(H5N1)) hemaggl...
142. MH165820 Influenza A virus (A/chicken/South Africa/451457/2017(H5N8)) segmen...
143. MN252530 Influenza A virus (A/chicken/South Africa/499723/2018(H5N8)) segmen...
144. CY020677 Influenza A virus (A/chicken/Sudan/2115-12/2006(H5N1)) segment 4 co...
145. KU646909 Influenza A virus (A/chicken/Taiwan/01174/2015(H5N3)) segment 4 hem...
146. KJ162708 Influenza A virus (A/chicken/Taiwan/4443/2013(H5N2)) segment 4 hemag...
147. KY989969 Influenza A virus (A/chicken/Taiwan/CH-A1029/2010(H5N2)) segment 4 ...
148. KY989967 Influenza A virus (A/chicken/Taiwan/CY-A2628/2012(H5N2)) segment 4 h...
149. MN988805 Influenza A virus (A/chicken/Taiwan/D9/2019(H5N2)) segment 4 hemag...
150. CY034683 Influenza A virus (A/chicken/Texas/167280-4/2002(H5N3)) segment 4 se...
151. GU052644 Influenza A virus (A/chicken/Texas/298313-2/2004(H5N2)) segment 4 h...
152. EF467802 Influenza A virus (A/chicken/Thailand/2/04(H5N1)) segment 4 hemaggluti...
153. EU497921 Influenza A virus (A/chicken/Thailand/ICRC-213/2007(H5N1)) hemaggluti...
154. EU547798 Influenza A virus (A/chicken/Thailand/ICRC-618/2008(H5N1)) hemaggluti...
155. HM590797 Influenza A virus (A/chicken/Thailand/ICRC-7372/2010(H5N1)) segment ...
156. JQ794462 Influenza A virus (A/chicken/Thailand/TS03/2006(H5N1)) segment 4 hem...


Consensus
P6
P5
P4
P3
P2
P1
331
Pro Gln Arg Glu Arg Arg Arg Lys Lys Arg Gly
127. EU148404 Influenza A virus (A/chicken/Nigeria/1071-10/2007(H5N1)) hemagglutini...
128. AM262542 Influenza A virus (A/chicken/Nigeria/BA210/2006(H5N1)) partial HA gen...
129. KF638579 Influenza A virus (A/chicken/Ningxia/222/2012(H5N1)) segment 4 hemag...
130. AB188816 Influenza A virus (A/chicken/Oita/8/2004(H5N1)) HA gene for hemaggluti...
131. KP739405 Influenza A virus (A/chicken/Oregon/41613-2/2014(H5N8)) segment 4 h...
132. CY068910 Influenza A virus (A/chicken/Pakistan/SPVC-8/2007(H5N1)) segment 4 se...
133. JF827081 Influenza A virus (A/chicken/Pakistan-Khi/NARC-612/2008(H5N1)) segme...
134. GU049896 Influenza A virus (A/chicken/Pennsylvania/446080-7/2006(H5N2)) segm...
135. FM163440 Influenza A virus (A/chicken/Poland/R3248/2007(H5N1)) HA gene for he...
136. FJ654295 Influenza A virus (A/chicken/Primorsky/85/2008(H5N1)) segment 4 hema...
137. EU332353 Influenza A virus (A/chicken/Romania/TL/nov/2007(H5N1)) hemagglutini...
138. KM190930 Influenza A virus (A/chicken/Scotland/1959(H5N1)) segment 4 hemagglu...
139. GU354081 Influenza A virus (A/chicken/Sikkim/151464/2009(H5N1)) segment 4 he...
140. KY614832 Influenza A virus (A/chicken/Sleman/04141596/2014(H5N1)) segment 4 ...
141. CY091966 Influenza A virus (A/chicken/Sleman/BBVW-82-65/2008(H5N1)) hemaggl...
142. MH165820 Influenza A virus (A/chicken/South Africa/451457/2017(H5N8)) segmen...
143. MN252530 Influenza A virus (A/chicken/South Africa/499723/2018(H5N8)) segmen...
144. CY020677 Influenza A virus (A/chicken/Sudan/2115-12/2006(H5N1)) segment 4 co...
145. KU646909 Influenza A virus (A/chicken/Taiwan/01174/2015(H5N3)) segment 4 hem...
146. KJ162708 Influenza A virus (A/chicken/Taiwan/4443/2013(H5N2)) segment 4 hemag...
147. KY989969 Influenza A virus (A/chicken/Taiwan/CH-A1029/2010(H5N2)) segment 4 ...
148. KY989967 Influenza A virus (A/chicken/Taiwan/CY-A2628/2012(H5N2)) segment 4 h...
149. MN988805 Influenza A virus (A/chicken/Taiwan/D9/2019(H5N2)) segment 4 hemag...
150. CY034683 Influenza A virus (A/chicken/Texas/167280-4/2002(H5N3)) segment 4 se...
151. GU052644 Influenza A virus (A/chicken/Texas/298313-2/2004(H5N2)) segment 4 h...
152. EF467802 Influenza A virus (A/chicken/Thailand/2/04(H5N1)) segment 4 hemaggluti...
153. EU497921 Influenza A virus (A/chicken/Thailand/ICRC-213/2007(H5N1)) hemaggluti...
154. EU547798 Influenza A virus (A/chicken/Thailand/ICRC-618/2008(H5N1)) hemaggluti...
155. HM590797 Influenza A virus (A/chicken/Thailand/ICRC-7372/2010(H5N1)) segment ...
156. JQ794462 Influenza A virus (A/chicken/Thailand/TS03/2006(H5N1)) segment 4 hem...
157. MH569621 Influenza A virus (A/chicken/Uganda/17RS115-15/2017(H5N8)) segmen...
158. CY105894 Influenza A virus (A/chicken/Ukraine/AV453/2008(H5N1)) hemagglutinin ...
159. MT547570 Influenza A virus (A/chicken/Viet Nam/HU12-1328/2019(H5N1)) segmen...
160. EF566200 Influenza A virus (A/Chicken/Viet Nam/NCVD09/2005(H5N1)) segment 4 ...
161. MK963403 Influenza A virus (A/chicken/Viet Nam/QN-0271/2013(H5N2)) segment ...
162. CY099968 Influenza A virus (A/chicken/Viet Nam/TMU015/2008(H5N1)) hemaggluti...
163. CY095701 Influenza A virus (A/chicken/Viet Nam/TMU025/2009(H5N1)) hemaggluti...
164. EU118132 Influenza A virus (A/chicken/Vietnam/G04/2004(H5N1)) segment 4 hema...
165. LC194831 Influenza A virus (A/chicken/Vietnam/HU3-83/2015(H5N1)) HA gene for ...
166. KY171732 Influenza A virus (A/chicken/Vietnam/NCVD-15A59/2015(H5N6)) segmen...
167. KR732552 Influenza A virus (A/chicken/Vietnam/NCVD-421/2010(H5N1)) segment 4...
168. KY171509 Influenza A virus (A/chicken/Vietnam/NCVD-1104/2013(H5N1)) segment ...
169. KY171350 Influenza A virus (A/chicken/Vietnam/NCVD-1982/2012(H5N1)) segment ...
170. KP097925 Influenza A virus (A/chicken/Vietnam/NCVD-A937/2011(H5N1)) segment ...
171. LC376799 Influenza A virus (A/chicken/Vietnam/QuangBinh/BT1113/2017(H5N6)) ...
172. KU143255 Influenza A virus (A/chicken/Wenzhou/HAYXLG03/2015(H5N2)) segment...
173. KU143256 Influenza A virus (A/chicken/Wuhan/WHJF/2014(H5N2)) segment 4 hema...
174. KU143267 Influenza A virus (A/chicken/Wuhan/WHYJ01/2015(H5N6)) segment 4 he...
175. JQ936687 Influenza A virus (A/chicken/Yangon/254/2010(H5N1)) segment 4 hemag...
176. MF973225 Influenza A virus (A/chicken/Zimbabwe/AI4935/2017(H5N8)) segment 4 ...
177. KJ509124 Influenza A virus (A/Korean native chicken/Korea/H257/2014(H5N8)) seg...
178. MT027086 Influenza A virus (A/layer hen/Slovakia/A-chicken-Slovakia-Pah 14-2020 ...

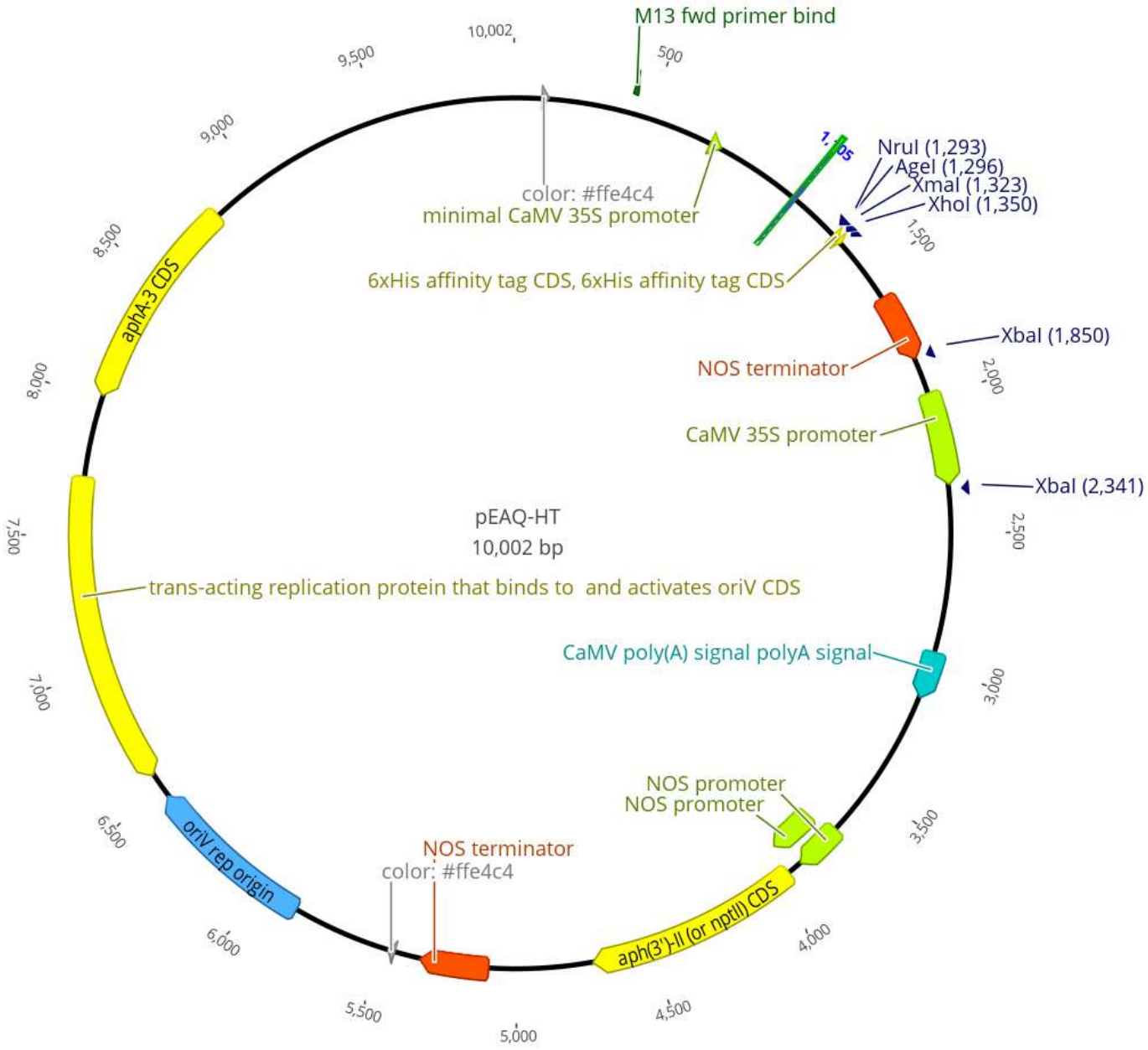
Appendix H. Cleavage site analysis of H9N2 worldwide




P6 P5 P4 P3 P2 P1 321
Consensus Pro Ala Arg Ser Ser Arg Gly
53. GQ497132 Influenza A virus (A/chicken/Iran/119/2005(H9N2)) segment 4 hemaggluti...
54. GQ497130 Influenza A virus (A/chicken/Iran/152/2004(H9N2)) segment 4 hemaggluti...
55. JQ419724 Influenza A virus (A/chicken/Iran/192/2009(H9N2)) segment 4 hemaggluti... Lys
56. GQ497123 Influenza A virus (A/chicken/Iran/233/2001(H9N2)) segment 4 hemaggluti...
57. GQ497121 Influenza A virus (A/chicken/Iran/565/2000(H9N2)) segment 4 hemaggluti...
58. GQ497117 Influenza A virus (A/chicken/Iran/698/1998(H9N2)) segment 4 hemaggluti...
59. GQ497120 Influenza A virus (A/chicken/Iran/772/1999(H9N2)) segment 4 hemaggluti...
60. EF063734 Influenza A virus (A/chicken/Iran/B11A/2005(H9N2)) hemagglutinin (HA) g...
61. EF063731 Influenza A virus (A/chicken/Iran/B76/2004(H9N2)) hemagglutinin (HA) ge...
62. EF063727 Influenza A virus (A/chicken/Iran/B314/2003(H9N2)) hemagglutinin (HA) g...
63. JN646748 Influenza A virus (A/chicken/Iran/EBGV-88/2010(H9N2)) segment 4 hemag... Lys
64. KJ696533 Influenza A virus (A/chicken/Iran/Markazi/2013(H9N2)) segment 4 hemaggl... Lys
65. KJ696534 Influenza A virus (A/chicken/Iran/Markazi/2014(H9N2)) segment 4 hemaggl... Lys
66. JX456177 Influenza A virus (A/chicken/Iran/SS3/2008(H9N2)) segment 4 hemagglutini...
67. JX456182 Influenza A virus (A/chicken/Iran/SS8/2011(H9N2)) segment 4 hemagglutini... Lys
68. EU477247 Influenza A virus (A/Chicken/Iran/TH78/1999(H9N2)) hemagglutinin (HA) g...
69. EU477248 Influenza A virus (A/Chicken/Iran/TH79/2000(H9N2)) hemagglutinin (HA) g...
70. EU477246 Influenza A virus (A/Chicken/Iran/TH81/2002(H9N2)) hemagglutinin (HA) g...
71. GU071984 Influenza A virus (A/chicken/Iran/THLBM868/2007(H9N2)) segment 4 he... Asn Lys Arg
72. MK159013 Influenza A virus (A/chicken/Iran/UT-Barin/2017(H9N2)) segment 4 hema... Lys
73. JX465626 Influenza A virus (A/chicken/Iran/ZMT-101/1998(H9N2)) segment 4 hemag...
74. MH879789 Influenza A virus (A/chicken/Iraq/3/2016(H9N2)) segment 4 hemagglutini... Lys
75. KJ831058 Influenza A virus (A/chicken/Israel/14/2014(H9N2)) segment 4 hemagglutin... Pro
76. JQ254937 Influenza A virus (A/chicken/Israel/32/2009(H9N2)) segment 4 hemaggluti...
77. JQ973656 Influenza A virus (A/chicken/Israel/58/2012(H9N2)) segment 4 hemaggluti...
78. KF918709 Influenza A virus (A/chicken/Israel/74/2013(H9N2)) segment 4 hemaggluti...
79. GQ120553 Influenza A virus (A/chicken/Israel/375/2007(H9N2)) segment 4 hemagglu...
80. KX783420 Influenza A virus (A/chicken/Israel/450/2016(H9N2)) segment 4 hemagglut...
81. EF492231 Influenza A virus (A/chicken/Israel/786/2001(H9N2)) hemagglutinin (HA) g... Asn
82. EF492236 Influenza A virus (A/chicken/Israel/909/2005(H9N2)) hemagglutinin (HA) g...
83. GQ120561 Influenza A virus (A/chicken/Israel/1040/2007(H9N2)) segment 4 hemaggl...
84. JQ254942 Influenza A virus (A/chicken/Israel/1067/2010(H9N2)) segment 4 hemaggl...
85. JQ973658 Influenza A virus (A/chicken/Israel/1115/2011(H9N2)) segment 4 hemaggl... His
86. JQ973661 Influenza A virus (A/chicken/Israel/1205/2008(H9N2)) segment 4 hemaggl...
87. EF492240 Influenza A virus (A/chicken/Israel/1475/2003(H9N2)) hemagglutinin (HA) ... Thr
88. FJ464729 Influenza A virus (A/chicken/Israel/1548/2006(H9N2)) segment 4 hemagglu...
89. EF492221 Influenza A virus (A/chicken/Israel/90658/2000(H9N2)) hemagglutinin (HA)...
90. MH558949 Influenza A virus (A/chicken/Israel/280662/2017(H9N2)) segment 4 hema... His Asn
91. MK942499 Influenza A virus (A/chicken/Israel/314616/2018(H9N2)) segment 4 hema...
92. MT499385 Influenza A virus (A/chicken/Israel/318798/2019(H9N2)) segment 4 hema...
93. JX273544 Influenza A virus (A/chicken/Israel/421201/2004(H9N2)) segment 4 hemag...
94. LC208476 Influenza A virus (A/chicken/Japan/AQ-HE61/2015(H9N2)) HA gene for he... Ser
95. MF673351 Influenza A virus (A/chicken/Jordan/56/2003(H9N2)) segment 4 hemagglu...
96. MF673311 Influenza A virus (A/chicken/Jordan/70/2004(H9N2)) segment 4 hemagglu...
97. MF673335 Influenza A virus (A/chicken/Jordan/88/2005(H9N2)) segment 4 hemagglu...
98. MN242766 Influenza A virus (A/chicken/Kenya/A71-1404/2017(H9N2)) segment 4 he... His
99. JX094859 Influenza A virus (A/chicken/Korea/01310 CE20/2001(H9N2)) segment 4 he... Thr Gly
100. KF188387 Influenza A virus (A/chicken/Korea/25232-96006/1996(H9N2)) segment ... Ala Tyr
101. KY785729 Influenza A virus (A/chicken/Korea/GS-3P/2005(H9N2)) segment 4 hemag... Thr Gly
102. JN852803 Influenza A virus (A/chicken/Korea/KNUWSJ09/2009(H9N2)) segment 4 h... Thr Gly
103. KT157805 Influenza A virus (A/chicken/Korea/KU178/2008(H9N2)) segment 4 hema... Ala Gly
104. AY790313 Influenza A virus (A/chicken/Korea/S1/2003(H9N2)) HA gene complete cds Ala Gly


Consensus
P6 Pro
P5 Ala
P4 Arg
P3 Ser
P2 Ser
P1 Arg
321 Gly
92. MT499385 Influenza A virus (A/chicken/Israel/318798/2019(H9N2)) segment 4 hema...
93. JX273544 Influenza A virus (A/chicken/Israel/421201/2004(H9N2)) segment 4 hemag...
94. LC208476 Influenza A virus (A/chicken/Japan/AQ-HE61/2015(H9N2)) HA gene for he... Ser
95. MF673351 Influenza A virus (A/chicken/Jordan/56/2003(H9N2)) segment 4 hemagglu...
96. MF673311 Influenza A virus (A/chicken/Jordan/70/2004(H9N2)) segment 4 hemagglu...
97. MF673335 Influenza A virus (A/chicken/Jordan/88/2005(H9N2)) segment 4 hemagglu...
98. MN242766 Influenza A virus (A/chicken/Kenya/A71-1404/2017(H9N2)) segment 4 he... His
99. JX094859 Influenza A virus (A/chicken/Korea/01310 CE20/2001(H9N2)) segment 4 he... Thr Gly
100. KF188387 Influenza A virus (A/chicken/Korea/25232-96006/1996(H9N2)) segment ... Ala Tyr
101. KY785729 Influenza A virus (A/chicken/Korea/GS-3P/2005(H9N2)) segment 4 hemag... Thr Gly
102. JN852803 Influenza A virus (A/chicken/Korea/KNUWSJ09/2009(H9N2)) segment 4 h... Thr Gly
103. KT157805 Influenza A virus (A/chicken/Korea/KU178/2008(H9N2)) segment 4 hema... Ala Gly
104. AY790313 Influenza A virus (A/chicken/Korea/S1/2003(H9N2)) HA gene complete cds Ala Gly
105. KF188395 Influenza A virus (A/chicken/Korea/S26/2004(H9N2)) segment 4 hemaggl... Ala Gly
106. KY785737 Influenza A virus (A/chicken/Korea/SL-4P/2002(H9N2)) segment 4 hemag... Thr Gly
107. JX273545 Influenza A virus (A/chicken/Kuwait/9/2004(H9N2)) segment 4 hemaggluti... Lys
108. MG882009 Influenza A virus (A/chicken/Lebanon/61/2017(H9N2)) segment 4 hema...
109. JX273546 Influenza A virus (A/chicken/Lebanon/1080/2004(H9N2)) segment 4 hem...
110. JX273551 Influenza A virus (A/chicken/Nepal/5430/2011(H9N2)) segment 4 hemagg... Lys
111. KF188299 Influenza A virus (A/chicken/Pakistan/2/1999(H9N2)) segment 4 hemaggl...
112. MH180388 Influenza A virus (A/chicken/Pakistan/22/2014(H9N2)) segment 4 hema... Lys
113. MG210366 Influenza A virus (A/chicken/Pakistan/103/2016(H9N2)) segment 4 hem... Lys
114. MH105306 Influenza A virus (A/chicken/Pakistan/304/2018(H9N2)) segment 4 hem... Lys
115. MG720815 Influenza A virus (A/chicken/Pakistan/871CF/2015(H9N2)) segment 4 he... Lys
116. JQ905259 Influenza A virus (A/chicken/Pakistan/MM81/2010(H9N2)) segment 4 he... Lys
117. KF188325 Influenza A virus (A/chicken/Pakistan/NARC-1617/2005(H9N2)) segment ...
118. JX273554 Influenza A virus (A/chicken/Pakistan/NARC-2434/2006(H9N2)) segment ...
119. CY038466 Influenza A virus (A/chicken/Pakistan/UDL-02/2008(H9N2)) segment 4 se... Lys
120. CY038482 Influenza A virus (A/chicken/Pakistan/UDL-04/2007(H9N2)) segment 4 se...
121. JX273556 Influenza A virus (A/chicken/Saudi Arabia/582/2005(H9N2)) segment 4 he...
122. CY081264 Influenza A virus (A/chicken/Saudi Arabia/CP7/1998(H9N2)) hemagglutini...
123. JX273558 Influenza A virus (A/chicken/Saudi Arabia/D-36363/2010(H9N2)) segment... His
124. GU050554 Influenza A virus (A/chicken/Saudi Arabia/EPD-22-01/2002(H9N2)) segm...
125. JX273560 Influenza A virus (A/chicken/Saudi Arabia/F-36365/2006(H9N2)) segment ...
126. KC767259 Influenza A virus (A/chicken/Shaanxi/11/2012(H9N2)) segment 4 hemagg... Ser
127. JF323006 Influenza A virus (A/chicken/Tunisia/12/2010(H9N2)) segment 4 complete...
128. MG881999 Influenza A virus (A/chicken/Uganda/MUWRP-201245/2017(H9N2)) seg... His
129. MN176651 Influenza A virus (A/chicken/Viet Nam/3DO1/2017(H9N2)) segment 4 he... Ser
130. MK964275 Influenza A virus (A/chicken/Viet Nam/HN-1728/2014(H9N2)) segment 4... Ser
131. MK964544 Influenza A virus (A/chicken/Viet Nam/QN-0576/2013(H9N2)) segment 4... Ser
132. MH560067 Influenza A virus (A/chicken/Vietnam/NCVD-15A13/2015(H9N2)) segme... Ser
133. AB256714 Influenza A virus (A/chicken/Yokohama/aq134/2002(H9N2)) HA gene for...
134. AB256746 Influenza A virus (A/chicken/Yokohama/aq144/2001(H9N2)) HA gene for...
135. KY910830 Influenza A virus (A/Egypt/ZU63/2016(H9N2)) segment 4 hemagglutinin (...
136. EF620900 Influenza A virus (A/Korea/KBNP-0028/2000(H9N2)) segment 4 complete ... Ala Gly
137. JF917103 Influenza A virus (A/Korean native chicken/Korea/K040110/2010(H9N2)) s... Thr Gly
138. MN530440 Influenza A virus (A/korean native chicken/South Korea/N07-1242/2007... Ala Gly
139. MN530492 Influenza A virus (A/korean native chicken/South Korea/N11-086/2011(... Thr Gly
140. MN530499 Influenza A virus (A/korean native chicken/South Korea/N12-014/2012(... Thr Gly
141. MN530509 Influenza A virus (A/korean native chicken/South Korea/N13-023/2013(... Thr Gly
142. MN530510 Influenza A virus (A/korean native chicken/South Korea/N15-001/2015(... Thr Gly
143. LC534257 Influenza A virus A/Chicken/Egypt/CL79/2013 HA gene for hemagglutinin...

Appendix I. Plasmid map of pEAQ-HT vector.



Appendix J. Plasmid map of pHREAC vector

