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Truncated Sequences of Influenza A Virus Subtype H5 Haemagglutinin for Vaccination and Diagnostic Purposes

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Abbreviations-

aa	Amino acid
Ab	Antibody
Ac-Elisa	Antigenic capture Elisa
ACN	Acetonitrile
AE	Avian encephalomyelitis
AGID	Agar gel immunodiffusion test
AI	Avian influenza
AIV	Avian influenza virus
AOX	Alcohol oxidase
APS	Ammonium peroxidisulfate
As	Antisense
Asn	Asparagine
BSA	Bovine serum albumin
^{o}C	Centigrade
cDNA	Complementary DNA
c-Elisa	Commercial Elisa
Con A	Concanavalin A
CPE	Cytopathic effect
CTL	Cytotoxic lymphocyte
Da	Dalton
DAB	3, 3´Diaminobenzidin tetra- hydrochloride
dist. H2O	Distilled water
DIVA	Differentiation between infected and vaccinated animal
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DVE	Duck virus enteritis
DVH	Duck virus hepatitis
E-Coli	*Escherichia coli*

EDTA	Ethylenediaminetetraacetic acid
Elisa	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FITC	Fluorescence isothiocynate
FCS	Fetal calf serum
FP	Fowl pox
GluNAc	N- acetylglutamic acid
GMT	Geometric mean titre
h	Hour
HA	Haemaglutinatinin
HAU	Haemagglutination unit
His4	Histidinol dehydrogenase gene
HIV	Human immunodeficiency virus
HP	Highly pathogenic
HPAI	Highly pathogenic avian influenza
HRP	Horse radish peroxidase
IB	Infectious bronchitis
IBD	Infectious bursal disease
I-Elisa	Indirect Elisa
IFA	Indirect immunoflurescence assay
IFN	Interferon
Ig	Immunoglobulin
ILT	Infectious laryngotracheitis
IVPI	Intra-venous pathogenicity index
Kb	Kilobase (1000 base)
Kda	Kilodalton
LAT	Latex agglutination test
LP	Low pathogenic
LPAI	Low pathogenic avian influenza
LS	Low salt
M	Matrix
mAB	Monoclonal antibody
MALDI-TOF	Matrix assisted laser desorption/ ionisation time of flight
Man.	Mannose

MCS	Multiple cloning sites
MD	Marek’s disease
µl	Microlitre
mM	Milli molar
M.O.I.	Multiplicity of infection
NA	Neuraminidase
ND	Newcastle disease
NEP	Nuclear export signal
Ni-NTA	Nickel- nitrilotriacetic acid
NP	Nucleoprotein
NS	Non- structural protein
NT	Neutralization test
OD	Optical density
OIE	World organisation for animal health
PA	Polymerase A protein
PAGE	poly-acylamide gel electrophoresis
PB1	Polymerase B1 protein
PB2	Polymerase B2 protein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsalfonylfluorid
P. pastoris	*Pichia pastoris*
PV	Post vaccination
rElisa	Recombinant Elisa
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse transcriptase polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisae*
Ser	Serine
SDS	Sodium dodecylsulfate
ss-RNA	Single-stranded RNA
TCA	Trichloroacetic acid
$TCID_{50}$	Tissue culture infectious dose

TEMED	N,N,N´, N´- tetramethyl-ethylendiamine
Thr	Threonine
Tm	Melting temperature
U	Unit
UV	Ultraviolet
V	Volt
v/v	Volume per volume
vRNP	Viral ribonucleoprotein
w/v	Weight per volume
YPD	Yeast extract peptone dextrose medium
YP Gly	Yeast peptone glycerol
YP MeOH	Yeast peptone methanol
Zeo	Zeocin

1. INTRODUCTION

Avian influenza (AI) affects the respiratory, digestive and/or nervous system of many bird species. AI viruses are influenza A viruses belonging to the Orthomyxovirus family, and they are classified according to their pathogenicity and the antigenicity of the surface proteins haemagglutinin (HA) and neuraminidase (NA) of which 16 and 9 variants, respectively, are known to date (FOUCHIER et al. 2005). Viruses containing subtypes H5 and H7 are highly pathogenic in poultry and cause outbreaks of highly pathogenic AI (HPAI), with mortality rates reaching 100 % (WEBSTER et al. 1992). HA-specific antibodies are protective as a result of their ability to prevent virus attachment and penetration of the host cell. HA is a homotrimer, each monomer is synthesised as a single polypeptide (HA0) that is cleaved by host proteases into HA1 and HA2. HA1 contains receptor-binding pocket surrounded by antigenic binding sites (BAIGENT and MCCAULEY 2003; KREIJTZ et al. 2007; NAYAK et al. 2009; SHOJI et al. 2008; TONEGAWA et al. 2003). H5 and H7 subtypes emerge at irregular intervals and cause severe economic losses in poultry (HAMPSON and MACKENZIE 2006), furthermore, it was demonstrated that H5N1 subtype could directly cross the species barrier to replicate in humans and cause severe disease (HAMPSON and MACKENZIE 2006; KODIHALLI et al. 2000). Vaccination can be a powerful tool to support eradication programs if used in conjunction with other control methods**.** Adjuvanted killed vaccines can provide a strong humoral immune response and they provide an effective protection against homologous low pathogenic AI (LPAI) and HPAI challenges. One of the concerns in the use of the commercially available vaccines (consisting of inactivated AI virus) to control HPAI in poultry farms is the possibility that while these vaccines may protect from disease, they do not hinder infection. Thus asymptomatic virus circulation may continue, resulting in spread of infection to non-immunized birds, e.g. in other (neighbouring) farms (KODIHALLI. et al. 2000). Inactivated influenza vaccines will lead to development of antibodies not only to the protective epitopes on the HA and NA, but also to the internal proteins which make the differentiation between infected and vaccinated animals (DIVA) difficult (SUAREZ and SCHULTZ-CHERRY 2000)**.** On the other hand, inactivated hetrologous vaccines are manufactured in a similar way to inactivated homologous ones. The use of hetrologous neuraminidase DIVA strategy is an acceptable approach but availability of diagnostics is an issue. Several recombinant fowl pox (FP) viruses expressing the H5 antigen have been developed

and one has been licensed in Mexico (SWAYNE and SUAREZ 2000). Other vectors have been used to successfully deliver the H5 or H7 antigens, such as constructs using infectious laryngotracheitis virus (ILT). Recombinant vectored vaccines also enable DIVA. However, their use is restricted to countries in which they are legally available. In addition, the use of these vaccines is also restricted to species in which the vector virus will replicate (SWAYNE et al. 2000). Peptide vaccination could be an alternative to commercially available vaccines. Subunit vaccines based on conserved antigens provide broader protection. Moreover, HA protein derived recombinant peptides would not elicit an immune response against internal viral proteins which facilitate DIVA. The HA1 antigenic domain of HA has been shown to induce an immune response equal to that of the full-size protein (TONEGAWA et al. 2003). Unfortunately, there is no effective and specific treatment of HPAI in poultry. The precise diagnosis of AI and effective vaccination, which has been shown to induce immune responses, can help control the spread of the disease. Hence, the on-site and rapid detection of AIV and surveillance of AI in flocks is significant for the economics of poultry production and human health (BECK et al. 2003). Flocks are usually tested as a group, rather than testing all the individual birds. Often ten to thirty birds are randomly selected from suspect flock and the birds are tested with a type-specific influenza detection test. Serological methods are usually used for detection of type-specific antibodies produced against nucleoprotein antigen (NP) and subtype antibodies against HA and NA. Agar gel immunodiffusion test (AGID) is used to detect circulating antibodies to type A influenza group-specific antigens, namely the NP and Matrix (M), regardless of subtype. It is preferred for its simple and fast realization and for the possibility of studying large numbers of samples. AGID test may not be suitable as a universal assay for some other species of birds; serum samples from water fowl do not contain good precipitin antibodies (CATTOLI et al. 2006; SUAREZ and SCHULTZ-CHERRY. 2000)**.** The HI test is more sensitive and rapid than the AGID test. However, it is allowed the titration of antibodies and it is one of the best technique to measure the level of protection in vaccinated chickens as well as to check the efficacy of vaccine (MEULEMANS et al. 1987). However, it is complicated due to the existence of 16 HA subtypes of AIV and it is laborious.

The indirect Elisa (I-Elisa) using crude or purified viral antigen on the solid-phase to detect viral specific antibodies has been developed for detection of chicken and turkey antibodies to AIV (ABRAHAM et al. 1988; ADAIR et al. 1989). Several different

types of Elisa have been developed for chicken (ZHOU et al. 1998). The Elisa rely upon the detection of antibodies against NP (BECK et al. 2003). Recently, detection of NP, N3 and N7 antibodies to AI virus by indirect ELISA using yeast-expressed antigens revealed that these indirect Elisas are rapid, sensitive, specific and can be used as promising tests during serological surveillance (UPADHYAY et al. 2009). Furthermore, the recombinant protein-based serological tests may have higher sensitivity and specificity as the target antigen is immuno-dominant and devoid of any non-specific moities present in whole cell preparations (ERRINGTON et al. 1995; MOHAN et al. 2006). The yeast *P. pastoris* has the potential of rapid growth to very high cell densities in inexpensive media as strong promoters are available (ROMANOS et al. 1992). It can produce high-level of foreign proteins either intracellular or extracellular. In addition, it has the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing (CEREGHINO et al. 2002; DALY and HEARN. 2005).

Aim of the work:

1. Expression of truncated sequences of influenza A subtype H5N1 in *P. pastoris*
2. Studying the possibilities to be used for immunization of chickens against H5 influenza virus
3. Development of recombinant Elisa for detection of influenza A subtypes H5 antibodies in chickens and ducks.

2. REVIEW OF LITERATURE

2.1 Avian influenza virus

AI, an infectious disease of birds that is caused by influenza virus type A strains, was identified first in Italy in 1878 (LIGON 2005)**.** The causative agent was eventually isolated from chickens in 1902 (A / Chicken / Brescia / 1902, H7N7). Similar outbreaks were observed in Europe and then worldwide, with subsequent isolation of several fowl plague viruses (H7 subtypes). By contrast, the first human influenza virus was not isolated until 1933 (LAMB and TAKEDA 2001)**.** Influenza virus was named in the 1960s because of their ability to bind to mucus and to distinguish them from another family of enveloped negative-strand RNA viruses (*paramyxoviridae)*. However, influenza viruses belong to *Orthomyxoviridae,* a Greek word (orthos, "standard, correct" and myxo, "mucus"). *Orthomyxoviruses* appear as roughly spherical or filamentous particles 80–120 nm in diameter or cross-section. The *Orthomyxoviridae* are composed of about 1 % RNA, 70 % protein, 20 % lipid, and 5 % to 8 % carbohydrate (LAMB and TAKEDA 2001)**.** At present the *Orthomyxoviridae* family consists of five genera: *influenzavirus A*, *influenzavirus B*, *influenzavirus C*, *ogotovirus*, tick-borne viruses that occasionally infect mammals, and *isavirus,* the virus responsible for infectious salmon anaemia. These viruses are enveloped RNA with single-stranded genomes of negative sense (i.e. the virus RNA is complementary to the messenger RNA (ALEXANDER 2006, 2007). Influenza viruses are polymorphic particles with a host-derived lipid bilayer envelope covered by about 500 projecting glycoprotein spikes with HA and NA activities (PEREZ et al. 2005). The viral genome of influenza A viruses consists of eight segments (Figure 1). To be infectious, a single virus particle must contain each of the eight unique RNA segments. Influenza A viral RNA segments encode 11 proteins as follow: polymerase B1 protein (PB1), polymerase B2 protein (PB2), polymerase A protein (PA), HA, NP, NA, matrix protein (M1), M2, non-structural 1 protein (NS1), NS2 and polymerase B1-F2 protein (PB1-F2) (SWAYNE 2006a; WEBSTER et al. 1992). Influenza viruses are classified into types A, B, and C on the basis of the antigenic nature of M1 and NP proteins (CHEN and DENG 2009; SUAREZ and SCHULTZ-CHERRY 2000). Type B and C viruses generally only infect humans, but type A viruses infect humans, pigs, horses, mink, marine mammals and a wide variety of domesticated and wild birds (OLSEN et al. 2006; TAMURA et al. 2005). Type A viruses are further subdivided into subtypes based on the antigenic differences in the HA and NA molecules. At present,

there are 16 HA (H1–H16) and NA (N1–N9) subtypes (FOUCHIER et al. 2005). Each virus has one H and one N subtypes. All H and N subtypes of influenza A viruses in the majority of possible combinations have been isolated from avian species (ALEXANDER 2000; TAMURA et al. 2005).

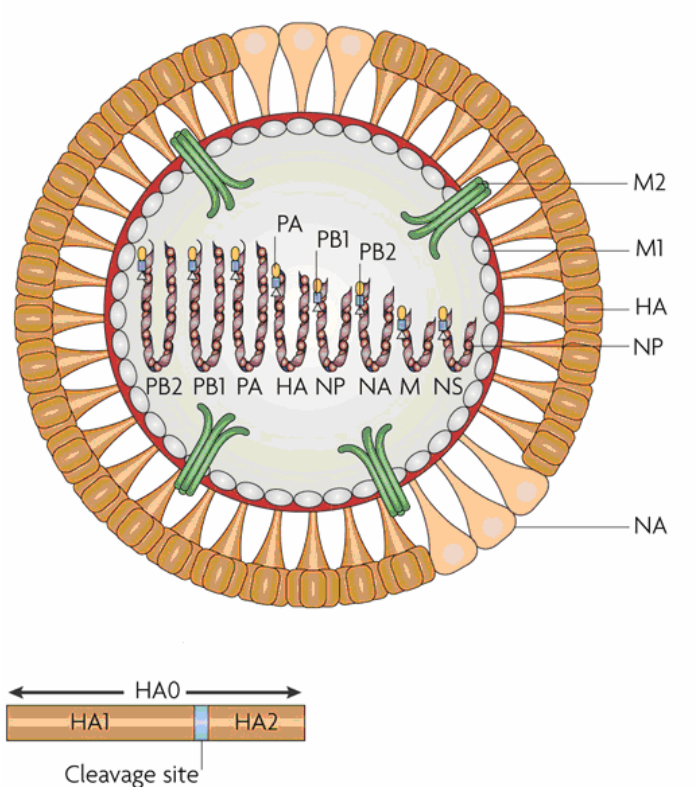


Figure (1): Schematic diagram of influenza A virus (SUBBARAO and JOSEPH 2007)

2.2 Influenza virus proteins

2.2.1 Polymerase subunits PB2, PB1, PA

PB2 polymerase is encoded by RNA segment 1, the slowest-migrating RNA species by gel electrophoresis. It is a member of the protein complex providing viral RNA-dependent RNA polymerase activity. It is known to function during initiation of viral mRNA transcription as this protein recognizes and binds the 5' cap structures of host cell mRNAs to use as viral mRNA transcription primers. Endonucleolytic cleavage of these cap structures from host mRNAs is also at least in part a function of PB2. The role of PB2 in the other virus-directed RNA synthetic processes (WEBSTER et al. 1992). PB1 polymerase is encoded by RNA segment 2. It functions in the RNA

polymerase complex as the protein responsible for elongation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis. PB1 proteins localizes in the nucleus of infected cells. Moreover, PA polymerase is encoded by RNA segment 3. It also localizes in the infected cell nucleus and is a member of the RNA-dependent RNA polymerase complex along with PB1 and PB2, but its role in viral RNA synthesis is unknown. There is evidence for possible roles as a protein kinase or as a helix-unwinding protein (WEBSTER et al. 1992)**.**

2.2.2 Nucleoprotein

NP is the major structural protein that interacts with the RNA segments to form ribonucleoprotein (RNP). NP is one of the type specific antigens and also the major target of cross reaction cytotoxic T lymphocytes generated against all influenza virus subtypes in mice and humans (LATHAM and GALARZA 2001)**.** NP functions in both viral assembly and RNA synthesis (BROWN 2000)**.**

2.2.3. Haemagglutinin

The HA was originally named because of the ability of the virus to agglutinate erythrocytes (LAMB and CHOPPIN 1983). The HA protein is an integral membrane protein and the major surface antigen of the influenza virus. It is responsible for binding to host cell receptors and for fusion between the virion envelope and the host cell. It undergoes three kinds of post translational processing; proteolytic cleavage, glycosylation, and fatty acid acylation. Newly synthesized HA is cleaved to remove the amino-terminal hydrophobic sequence of 14 to 18 amino acids (aa), which are the signal sequence for transport to the cell membrane. Carbohydrate side chains are added, whose number and position vary with the virus strain. The HA (HA0) is cleaved into two disulfide-linked chains, HA1 and HA2 (MITNAUL et al. 2000; SKEHEL and WILEY 2000)**.** This cleavage is accomplished by host-produced trypsin-like proteases (KIDO et al. 1992) and is required for infectivity because virus-cell fusion is mediated by the free amino terminus of HA2 (LEWIS 2006; STEVENS et al. 2006). The fully processed HA consists of HA1 (typically) of about 324 aa plus variable carbohydrate, and HA2 (typically) of about 222 aa plus variable carbohydrate and 3 palmitate residues. The three-dimensional structure of a complete HA trimer has been determined. Each HA molecule consists of a globular head on a stalk. The head is made up exclusively of HA1 and contains the receptor-binding cavity as well as most of the antigenic sites of the molecule. The stalk consists of all of HA2 and part of HA1. The carboxy-terminal region of HA2 contains the hydrophobic

transmembrane sequence and a terminal cytoplasmic anchor sequence where palmitate is attached (WEBSTER et al. 1992). The HA mediates attachment to and entry of virus into host cells by binding to sialic acid receptors at the cell surface. HA is also the main viral target of protective humoral immunity by neutralizing antibody (DE JONG and HIEN 2006; STEVENS et al. 2006) and the T - cell mediated immune responses (HORVATH et al. 1998). The binding affinity of HA to the sialic acid residues partly accounts for the host specificity of the various influenza A virus subtypes. The epitopes involved in receptor-binding show great variability due to mutations in the RNA causing amino acid substitutions at several sites on the HA1 molecule (BROWN 2000; HORVATH et al. 1998). Influenza viruses from different hosts can differ with respect to specificity and affinities for the 2-3 or 2-6 linkage of N-acetyl neuraminyl-galactose (SA 2,3 Gal; SA 2,6 Gal). These binding specificities correspond to the types of sialic acid linkages within these hosts and therefore avian strains preferentially bind SA 2,3 Gal, whereas human strains preferentially bind SA 2,6 Gal (WOOD et al. 1993). Owing to error-prone viral RNA polymerase activity, influenza virus HA is subject to a very high rate of mutation. Selection for amino acid substitution is driven at least in part by immune pressure, as the HA is the major target of the host immune response. Several recent reports demonstrated that HA and HA1 fragment containing the majority of antigenic determinants are responsible for generation of virus-neutralizing antibodies and vaccines based on conserved antigens provide broader protection (KREIJTZ et al. 2007; SHOJI et al. 2008; TONEGAWA et al. 2003).

2.2.4 Neuraminidase

NA is also a type II integral membrane glycoprotein (MITNAUL et al. 2000) and a second major surface antigen of the virion. NA cleaves terminal sialic acid from glycoprotein or glycolipids. Thus, it is thought to be important in the final stages of release of new virus particles from infected cells, prevent the new virus particles agglutinating, thus increasing the number of free virus particle and hence spread of the virus from original site of infection (DE JONG and HIEN 2006). NA is glycosylated and possesses an amino-terminal hydrophobic sequence which functions both as signal for transport to the cell membrane and as transmembrane domain; it is not cleaved away. The distribution of NA has not been conclusively resolved; immunogold-labeling experiments suggest that the NA tetramers are not evenly distributed over the virion envelope, as is HA, but aggregate into patches or caps

(BROWN 2000). The complete three-dimensional structure of an NA tetramer, bound to antibody, has been determined. Like HA, NA is highly mutable with variant selection partly in response to host immune pressure. Nine subtypes of NA have been identified in nature; they are not serologically cross-reactive. Different variants of several subtypes are known (BROWN 2000; WEBSTER et al. 1992). Many studies have documented that influenza virus particles with low NA enzymatic activity cannot be efficiently released from infected cells, resulting in the accumulation of large aggregates of progeny virions on the cell surface (LIU et al. 1995; MITNAUL et al. 2000). Inhibition of this important function represents the most effective antiviral treatment strategy to date (LEWIS 2006). The substrate binding site is in the middle of the head of tetramer, which is attached by a stalk to the membrane. Substrate analogues have recently been introduced for therapy of influenza virus infection. Anti-drugs currently being used to treat infected patients are oseltamivir (Tamiflu).

2.2.5 Non-structural proteins

NS1 mRNA is collinear with the viral RNA. The NS1 protein forms a dimmer that inhibits the export of poly-A containing mRNA molecules from nucleus and is expressed in large amounts in influenza virus infected cells but it has not been detected in virions, hence the designation NS for non-structural. NS1 is a phosphoprotein, and the protein is found in infected cells associated with polysomes and also in the nuclease and nucleolus. The NS1 protein is required for virulence in mice (LIPATOV et al. 2005)**.** When NS1 is absent or altered, the virulence of influenza A viruses is highly attenuated. NS1 protein functions as a type I interferon (IFN) antagonist raise the question whether this protein can also increase viral pathogenicity in vivo (LIPATOV et al. 2005). The NS1 protein of influenza A virus associates with p53 and inhibits p53-mediated transcriptional activity and apoptosis (WANG et al. 2010). The NS2 was originally misnamed but is not thought to exist in virions and to form an association with the M1 protein. NS2 is a small molecule. NS2 functions in the nucleocytoplasmic export of RNP for assemble into virions and have been shown to posses a nuclear export signal. A new name has been proposed nuclear export protein (NEP) (ALEXANDER 2007).

2.2.6 Matrix protein

Segment 7 encodes two proteins, M1 and M2. The proteins encoded by these mRNAs share their initial 9 amino acids and also have a stretch of 14 amino acids in overlapping reading frames. The M1 protein is a highly conserved 252-amino acid

protein. It is the most abundant protein in the viral particle, lining the inner layer of the viral membrane and contacting the RNP core. M1 has been shown to have several functions including regulation of the nuclear export of viral RNP (vRNPs), both permitting the transport of vRNP particles into the nucleus upon infection and preventing newly exported vRNP particles from re-entering the nucleus (MARTIN and HELENIUS 1991). M1 may also be involved in the inhibition of viral transcription in the late stages of infection and regulation of the switch from replication to viral assembly (PEREZ and DONIS 1998; WAKEFIELD and BROWNLEE 1989; ZVONARJEV and GHENDON 1980). M1 binds RNA (SHA and LUO 1997; YE et al. 1999; YE et al. 1989), vRNPs (YE et al. 1999) and lipids (GREGORIADES and FRANGIONE 1981) dimerizes with other M1 molecules (HARRIS et al., 2001); and interacts with both the HA and NA proteins (ALI et al. 2000; ENAMI and ENAMI 1996). It is also involved in export to the cytoplasmic membrane, virus assembly, and budding (GOMEZ-PUERTAS et al. 2000; LATHAM and GALARZA 2001). It has been reported repeatedly that the virulence and growth of influenza viruses are influenced by changes in the internal proteins. M1 protein is a multifunctional protein which contributes to the control of virulence, growth, (ENAMI et al. 1993; SMEENK and BROWN 1994; YASUDA et al. 1994) and host specificity of influenza viruses (MURPHY et al. 1989). M2 is an integral membrane protein and a large number of M2 molecules are expressed at the plasma membrane of the influenza virus infected cell surface, with a ratio of approximately two M2 molecule per HA (ZEBEDEE and LAMB 1988). The 97-amino acid M2 protein is a homotetrameric integral membrane protein exhibits ion channel activity and is composed of 24 extracellular amino acids, and 54 cytoplasmic residues (BAUER et al. 1999; HOLSINGER and LAMB 1991; SUGRUE and HAY 1991; WANG et al. 1994). Disulfide bonds link the protein through cysteines located in the extracellular region (HOLSINGER and LAMB 1991). Amantadine and remantadine inhibit virus replication by blocking the ion channel formed by the M2 Protein. Certain mutations in the M gene lead to viruses that are resistant to antiviral drugs (positions 26, 27, 30, 31, and 34) within the transmembrane domain of M2 has been implicated in loss of sensitivity of M2 blockers (HAY et al. 1979; PINTO et al. 1992).

2.2.7 PB1-F2 protein

PB1-F2 is encoded by an open reading frame within an alternative reading frame of PB1 (CHEN et al. 2001). This 87-residue protein seems to participate in the induction

of apoptosis and also functions to kill host immune cells responding to influenza virus infections. This protein was shown to be non-essential for virus replication in vitro (SIDORENKO and REICHL 2004).

2.3 Virus replication

Influenza virus particles attach to sialic acid containing cellular receptors via the viral HA glycoprotein. Virions penetrate into the cell by a clathrin-dependent receptor-mediated endocytosis. Although several other entry pathways for influenza virus are also reported (SIECZKARSKI and WHITTAKER 2002) the endocytic pathway seems to be the most common. vRNPs are released from the endosome when the endosomal pH is decreased to 5.0, which activates viral M2 ion channels and allows protons to enter the interior of the virus particle. As a result, the viral M1 proteins undergo conformational changes, followed by the disruption of M1- vRNP interactions and acid-catalysed conformational rearrangements of HA proteins. As a consequence, viral and endosomal membranes fuse and individual vRNPs are released into the cellular cytoplasm. The import into the nucleus, through nuclear pore complexes, is mediated by a nuclear localization signal (NLS), carried by NP. Since M1 proteins inhibit this import of vRNPs, its detachment from vRNP plays a crucial role at this step. Virus particles unable to fuse with the membrane, e.g., virions with defective M2 ion channels, are degraded by lysosomes. Three types of viral RNAs are synthesized in the cellular nucleus: viral mRNAs of positive polarity (vmRNA), viral genomic RNAs (vRNA) of negative polarity, and complementary RNAs (cRNA) of positive polarity. Influenza virus vmRNAs contain a cap structure at the 5´ end and a poly (A) tail at the 3´ end, which are taken from cellular precursor mRNAs. Their synthesis is governed by the viral polymerase complex and comprises several steps. Splicing of M and NS mRNAs, also occurs in the nucleus. It is regulated by NS1 proteins. Newly synthesized viral mRNAs are efficiently exported from the nucleus into the cytoplasm via nuclear pores. Genome replication involves the synthesis of full-length vRNA (–) and cRNA (+) strands. While transcription is carried out by PB1 and PB2 proteins, genome replication requires PB1 and PA subunits of the polymerase complex (CASSETTI et al. 2001). Experiments show that NP proteins bind to elongating strands. It is also known that NP proteins promote the initiation of unprimed transcription and block the synthesis of viral mRNAs. The cRNAs serve as templates for vRNA synthesis, while newly replicated vRNAs are used for the production of further vmRNAs and cRNAs as well as for the assembly of

vRNP complexes. The proteins PB1, PB2, PA, NP, NS1, NS2, and M1 are produced in the cellular cytoplasm. When influenza virus particles internalize into the cell, the rate of cellular protein production slows down. The hypothesis that the translation machinery of infected cells efficiently processes only mRNAs possessing the viral untranslated region was recently challenged (CASSETTI et al. 2001)**.** However, there are three possible mechanisms for the inhibition of cellular protein synthesis (PARK and KATZE 1995). One of them involves the degradation of cellular precursor mRNAs in the nucleus. Another possibility is the inhibition of the translation of cellular mRNAs at initiation and elongation steps. Finally, cellular protein production can be suppressed by retarding the transport of cellular mRNAs to the cytoplasm. While cellular protein synthesis is at least partially inhibited, viral proteins are synthesized at a maximum rate by ribosomes organized in polysome complexes. Newly synthesized polymerases as well as nucleocapsid, M, and NS are then transported to the nucleus, where they participate in M and NS mRNAs splicing, transcription, and genome replication. Additionally, they are consumed for the production of new vRNP complexes. M2, HA, and NA protein synthesis is carried out by ribosomes bound to the membranes of the endoplasmic reticulum (ER). Newly synthesized envelope proteins are inserted into the ER, glycosylated, and transported to the Golgi apparatus. Finally, they are delivered to the membrane of the host cell to be assembled with vRNP complexes. Formation of vRNP complexes takes place in the nucleus. It results from the binding of newly synthesized PB1, PB2, PA, NP, and NS2 proteins to vRNAs. M1 proteins attach to vRNPs, forming M1-vRNP complexes, and catalyze the transport of vRNPs to the cytoplasm. Nuclear export of vRNPs is also directed by NS2 proteins and nuclear export signals (NES) carried by NP proteins (PORTELA and DIGARD 2002). As stated above, M1 proteins also inhibit the import of vRNP complexes. Therefore, newly synthesized vRNPs associated with M1 protein are unable to penetrate into the nucleus again. The vRNP-M1 protein complexes interact with the cytoplasmic tails of M2, HA, and NA proteins, that leads to the formation of a bud at the assembly site, e.g., the apical membrane of polarized epithelial cells. This bud separates from the cellular membrane and a virion is released to the extracellular medium. Most of the cellular membrane proteins are excluded from virus particles by NA proteins (LUO et al. 1993)**.**

2.4 Antigenic shift and drift

Two surface glycoproteins HA and NA, undergo gradual, continuous minor antigenic changes due to point mutations in the HA and NA genes, referred to antigenic drift. This phenomenon occurs in all influenza A viruses due to the lack of a proof reading system for the RNA polymerases. Due to the segmented nature of the viral genome, the exchange of RNA segments, called genetic reassortment, between two genetically different AI viruses infecting the same host cell may potentially result in the generation of a novel strain and / or subtype, which can lead to major antigenic changes in the HA or NA genes. This is referred antigenic shift. The emergence of resultant virus strains enhanced human infectivity and may lead to influenza pandemics (BOUVIER and PALESE 2008; SHOHAM 2006; TAMURA et al. 2005; WEBSTER et al. 1992). Animals, particularly the importance of pigs and poultry have a great role in the emergence of the new influenza viruses (MANUGUERRA and HANNOUN 1997). On the other hand, ducks are the "Trojan horses" of H5N1 influenza (KIM et al. 2009). It is generally accepted that wild duck species can spread HP H5N1 viruses, but there is insufficient evidence to show that ducks maintain these viruses and transfer them from one generation to the next.

2.5 Avian influenza pathogenicity

Influenza A viruses are divided into two groups on the basis of their ability to cause disease in chickens which are HPAI and LPAI (PANTIN-JACKWOOD and SWAYNE 2009). HPAI viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI. All other viruses (LPAI) cause mild symptoms, which nevertheless, may be exacerbated by other infections or environmental conditions resulting in a much more serious disease. A crucial role in AI pathogenicity is played by HA. This is because HA0 requires post-translational cleavage by host proteases before the protein is functional and the virus particles are infectious. It has been demonstrated that the HA0 precursor proteins of AIV of low virulence for poultry are limited to cleavage by host proteases such as trypsin and trypsin-like enzymes. Thus AIV remains restricted to replication at sites in the host where such enzymes are found, i.e. the respiratory and intestinal tracts. In contrast virulent viruses appear to be cleavable by (a) ubiquitous protease(s). The remains to be fully identified but appears to be one or more proprotein-processing subtilisin-related endoproteases of which furin is the leading candidate (STIENEKE-GROBER et al. 1992). This enables these viruses to replicate throughout the animal, damaging vital organs and tissues

which brings about disease and death in the infected bird. Comparisons of the amino-acid sequences at the HA0 cleavage site of AIV of high and low pathogenicity revealed that while viruses of low virulence have a single basic amino acid (arginine) at the site, all HPAI viruses possessed multiple basic amino acids (arginine and lysine) adjacent to the cleavage site either as a result of apparent insertion or apparent substitution (CHEN et al. 1998a; SENNE et al. 1996; VEY et al. 1992; WOOD et al. 1993). The potential for LPAI virus appearing in meat of infected chickens is negligible, while the potential for having HPAI virus in meat from infected chickens is high, but proper usage of vaccines can prevent HPAI virus from being present in meat (SWAYNE and BECK 2005)**.** Most HPAI viruses appear to have arisen as result of spontaneous duplication of purine triplets, which results in the insertion of basic amino acids at the HA0 cleavage site, and this occurs because of a transcription fault by the polymerase complex. However, this is clearly not the only mechanism by which HPAI viruses arise. Some appear to result from nucleotide substitution rather than insertion, whereas others have insertions without repeating nucleotides. H7N3 HPAI viruses show distinct and unusual cleavage site amino acid sequences (PASICK et al. 2005). The factors that bring about mutation from LPAI to HPAI are not known. In some instances, mutation seems to have taken place immediately after introduction to poultry from wild birds at the primary site, whereas in others, the LPAI virus has circulated in poultry, sometimes for months before mutating. The HPAI viruses do not show high virulence for all species of birds, and the clinical severity seen in any host appears to vary with both bird species and virus strain (ALEXANDER et al. 1978; ALEXANDER et al. 1986). In particular, ducks rarely show clinical signs as a result of HPAI infections, although there are reports that some of the Asian H5N1 viruses have caused disease (STURM-RAMIREZ et al. 2005). Ostriches (*Struthio camelus*) also appear to have an unusual clinical response to HPAI infection. The clinical condition caused by HPAI is very similar to that caused by LPAI viruses (CAPUA and ALEXANDER 2006). Despite the application of control measures in most countries, infections of HPAI H5N1 continue to occur, and in 2008, outbreaks in poultry were reported in Benin, Egypt, Germany, Iran, India, Thailand, Turkey, Ukraine, and Vietnam, whereas infections of wild birds were reported in China, Hong Kong, and the United Kingdom. The peak virus titres excreted and the time required for virus titres to reach a minimal chicken infectious dose may be the critical phenotypes influencing the transmissibility of highly pathogenicity AI viruses in

chickens (SUZUKI et al. 2010). Moreover, H6N2 viruses can infect both chickens and ducks, but based on the number of birds shedding virus and on histopathology, these viruses appear to be more adapted to chickens. Virus shedding, which could go unnoticed in the absence of clinical signs in commercial chickens, can lead to transmission of the virus among poultry (JACKWOOD et al. 2010). The OIE adopted the following criteria for the classification of an AIV as HP: (1) any influenza virus that is lethal for six, seven or eight of eight 4– to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free infective allantoic fluid; any virus that has an intravenous pathogenicity index (IVPI) greater than 1.2; (2) the amino acid sequence of the connecting peptide of the HA must be determined. If the sequence is similar to that observed for other highly pathogenic AI isolates, the isolate being tested will be considered to be highly pathogenic.

2.6 Clinical signs

Following an incubation period of usually a few days (rarely up to 21 days), depending upon the characteristics of the isolate, the dose of inoculum, the species, and age of the bird, the clinical presentation of AI in birds is variable and symptoms are fairly unspecific (ELBERS et al. 2005)**.** The symptoms following infection with LPAI may be as discrete as ruffled feathers, transient reductions in egg production or weight loss combined with a slight respiratory disease (BARR et al. 1986; CAPUA and MUTINELLI 2001). Some LP strains such as certain Asian H9N2 lineages, adapted to efficient replication in poultry, may cause more prominent signs and also significant mortality (BANO et al. 2003). Both 1918 and 2009 H1N1 influenza viruses behave as LPAI in gallinaceous poultry (BABIUK et al. 2010). LPAI (H4, H6 and H9) can infect and be shed by chickens and turkeys. However, detection is difficult because these viruses don’t cause clinical disease or mortality, but only induce mild microscopic lesions and exhibit poor seroconversion (MORALES et al. 2009).

In HPAI, the illness in chickens and turkeys is characterized by a sudden onset of severe symptoms and a mortality that can approach 100 % within 48 hours (SWAYNE and SUAREZ 2000). Spread within an affected flock depends on the form of rearing: in herds which are litter-reared and where direct contact and mixing of animals is possible, spread of the infection is faster than in caged holdings but would still require several days for complete contagion (CAPUA et al. 2000). Often, only a section of a stable is affected. Many birds die without premonitory signs so that

sometimes poisoning is suspected in the beginning (NAKATANI et al. 2005). It is worth noting, that a particular HPAI virus isolate may provoke severe disease in one avian species but not in another: in live poultry markets in Hong Kong prior to a complete depopulation in 1997, 20 % of the chickens but only 2.5 % of ducks and geese harboured H5N1 HPAIV while all other galliforme, passerine and psittacine species tested virus-negative and only the chickens actually showed clinical disease (SHORTRIDGE 1982). Individual birds affected by HPAI often reveal little more than severe apathy and immobility (KWON et al. 2005). Oedema, visible at feather-free parts of the head, cyanosis of comb, wattles and legs, greenish diarrhoea and laboured breathing may be inconsistently present. In layers, soft-shelled eggs are seen initially, but any laying activities cease rapidly with progression of the disease (ELBERS et al. 2005). Nervous symptoms including tremor, unusual postures (torticollis), and problems with co-ordination (ataxia) dominate the picture in less vulnerable species such as ducks, geese, and ratites (KWON et al. 2005)

2.7 Gross lesions

The appearance of gross lesions is variable depending on the virus strain, the length of from infection to death, and the age and species of poultry affected (KOBAYASHI et al. 1996; SWAYNE et al. 1997). In general, clinical signs, lesions and death have been seen with domestic poultry of the order galliformes, family phasianidae, but not for birds of the orders anseriformes or charadriiformes when infected with HPAI viruses. In most cases peracute infections with death (days one to two of infection), poultry have lacked visible gross lesions (HOOPER et al. 1995)**.** However, some strains, such as A / chicken / Hong Kong / 220 / 97 (H5NI) and A / chicken / Italy / 330 / 97 (H5N2) have caused severe lung lesions of congestion, haemorrhage and edema in chicken, such that excised tissue exuded serous fluid and blood (SUAREZ et al. 1998)**.** Edema of the brain has also been reported. During the acute stages of infection with death (days three to five post-infection) chickens have ruffled feathers, congestion and/or cyanosis of the comb and wattles and swollen heads, especially prominent from periorbital and intramandibular subcutaneous oedema (ACLAND et al. 1984; HOOPER et al. 1995; KOBAYASHI et al. 1996; SWAYNE et al. 1997)**.** Some viruses produced hyperaemia and edema of the eyelids, conjunctiva and trachea (BARR et al. 1986)**.** In birds which die, generalized congestion and haemorrhage may occur (HOOPER 1989). Lesions are common in the combs and wattles, especially in adult chicken, and include petechial-to-ecchymotic

haemorrhages, swelling from oedema and eventually depressed dark red-to-blue areas of ischemic necrosis as the result of vascular infarction. Subcutaneous haemorrhages and oedema may be present around the hock, on the shanks and feet and occasionally on feathered skin all over the body. Some HPAI viruses, such as A / Queretaro / 14588-19 / 95 (H5N2), commonly caused thickening of the skin over the distal legs with gelatinous oedema (SWAYNE et al. 1997). Petechial – to- ecchymotic haemorrhages may be present in multiple visceral organs or on the serosal surface, such as the epicardium of the heart, serosa of small intestine ,abdomintal fat, serosa of sternum, caecal tonsils, Meckel's diverticulum, Peyer's lymphoid patches of the small intestine, proventriculus around the glandular ducts or between glands, under the cuticle of the skeletal muscles primary lymphoid organs such as cloacal bursa and thymuses are severely atrophic, while the spleen may be normal in size or enlarged. Occasionally, spleens have white foci of necrosis and the pancreas may have red to light orange to brown mottling (HOOPER 1989)**.** Ruptured ova with yolk peritonitis have been reported in layers and broilers turkey breeders.

2.8 Vaccination

Vaccination has proven to be a powerful tool for control of H5N1 HPAI outbreaks. Vaccination increases the bird resistance to field virus transmission (CAPUA and MARANGON 2007a; VAN DER GOOT et al. 2005; ELLIS et al. 2004)**.** Vaccination and companion DIVA testing are highly recommended by OIE for control and prevention of HPAI (CAPUA and MARANGON 2007a). Wild birds and waterfowl play a potential role as reservoirs in AIV circulation and evolution (NORMILE 2005; OLSEN et al. 2006)**.** However, the global distribution and persistence of LPAI viruses in wild bird populations is not fully clear (WEBBY and WEBSTER 2003). Influenza viruses can have numerous antigenic subtypes and rapidly evolve due to constant gene mutation and reassortment. These factors contribute to the fact that AI is a disease difficult to be eradicated in some circumstances. An ideal AI vaccine would provoke an immune response that protects against disease and prevents infection (SWAYNE and KAPCZYNSKI 2008). Current commercially available vaccines will not prevent infection completely, but experimental and field studies have shown that properly used vaccines can accomplish multiple goals: (1) protect against clinical signs and death, (2) reduce shedding of field virus if vaccinated poultry become infected, (3) prevent contact transmission of field virus, (4) protect against challenges by low to high doses of field virus, (5) protect against a changing virus, and (6)

increase a bird's resistance to AI virus infection (SWAYNE 2006b). If possible, selection of strains for inclusion in poultry AI vaccines should be based on an analysis of the field challenge virus in comparison with the licensed vaccine strains, which may require some flexibility in registration processes to allow for new products and re-formulation of existing vaccines to facilitate timely adjustments of vaccine formulation. Unlike human influenza A vaccines where antigenic drift of the field virus requires changing vaccine strains every few years, antigenic drift of poultry influenza viruses has not required a similar frequent need to change the vaccine strain (SWAYNE and KAPCZYNSKI 2008). In one study, an H5 vaccine strain provided broad protection against diverse H5 HPAI viruses collected during 38 years and differing as much as 12 % in amino acid sequence of the HA gene. The closer the HA gene sequence similarity between vaccine and field viruses, however, the greater the reduction in challenge virus replication and shedding from the respiratory tract (SWAYNE and KAPCZYNSKI 2008). The use of a poorly matched vaccine can result in clinical disease and increased virus shedding when vaccinated poultry are naturally infected (SWAYNE 2006b). The duration of effective immunity will vary based on the number of doses given, age of bird at time of vaccination, antigen quantity in each dose of vaccine, and avian species. Using traditional whole virus inactivated oil-emulsion vaccines in chickens, peak HI titres are observed 4-6 weeks post vaccination (BRUGH et al. 1979), with the same work indicating a much lower seroconversion rate in turkeys. Additional studies in turkeys indicate two doses of inactivated vaccine are necessary to reduce virus shedding and replication to levels needed to reduce viral spread (KARUNAKARAN et al. 1987). The immunogenicity of vaccines is correlated to antigen mass, its formulation and the age of vaccination (DI TRANI et al. 2003). The different levels of immune responses are due to antigenic quality and contents as well as the adjuvant composition (CRISTALLI AND CAPUA 2007).

2.8.1 Inactivated whole virus vaccine

As early as 1971, inactivated oil emulsion vaccines were used to immunize chickens and turkeys against AI virus infection (ALLAN et al. 1971). Up to now, inactivated vaccines in chickens and turkeys against AI have been reported, subtypes H1 (ABRAHAM et al. 1988; SWAYNE et al. 2001), H5 (CRAWFORD et al. 1998; SWAYNE 2006a; SWAYNE et al. 2001), H6 (CARDONA et al 2006), H7 (CHERBONNEL et al. 2003; FATUNMBI et al. 1992; PHILIPPA et al. 2005) and H9

(PAN et al. 2009; SWAYNE 2006a). Inactivated vaccines are major vaccine type used in the poultry industry. They produce strong humoral immune response but they do not produce a strong mucosal immune response. In addition, Adjuvanted killed vaccines can provide a strong humoral immune response and they provide an effective protection against LPAI and HPAI challenges. The main disadvantage of inactivated full virus vaccines is that vaccinated birds will develop antibody not only to protective epitopes on the HA and NA proteins, but also to the internal influenza proteins as M1 and NP. Vaccinated birds could not be distinguished from naturally infected birds using the commonly used serological assays as Elisa and AGID (MARANGON et al. 2007; SUAREZ and SCHULTZ-CHERRY 2000). On the other hand, inactivated heterologous vaccines are manufactured in a similar way to inactivated homologous ones. The use of heterologous NA DIVA strategy is an acceptable approach but availability of diagnostics is an issue. Reverse genetics have been applied to develop improved vaccines against AI (LIU et al. 2003; NEUMANN et al. 2003; TIAN et al. 2005; WEBSTER et al. 2006). Conventionally prepared reassortant H5N1 vaccines developed by reverse genetics are currently being used in China and other Asiatic countries (SWAYNE. 2009; TIAN et al. 2005). In addition, H7 and H9 subtype vaccines developed by reverse genetics have been used experimentally (CHEN 2004; JOSEPH et al. 2008). These vaccines have similar performance to conventional inactivated vaccines. However the efficacy under field conditions is questionable. This technology does not solve the problem related to the egg-based production as production of vaccines in egg is cumbersome, lengthy, and costly (SWAYNE 2009; WANG et al. 2006).

2.8.2 Live recombinant vector-based vaccine

Recombinant vectored vaccines have been developed for poultry using viral vectors such as FP virus (SWAYNE et al. 2000): vaccinia virus (CHAMBERS et al. 1988; DE et al. 1988; YEWDELL et al. 1985), retrovirus (ALTSTEIN et al. 2006; BROWN et al. 1992; HUNT et al. 1988), adenovirus (GAO et al. 2006; Hoelscher et al. 2006; SARUKHAN et al. 2001), ND virus (DINAPOLI et al. 2010; DINAPOLI et al. 2007; GE et al. 2007; KIM and SAMAL 2010; NAKAYA et al. 2001; NAYAK et al. 2009; PARK et al. 2006; ROMER-OBERDORFER et al. 2008; SCHROER et al. 2009; STEEL et al. 2008; VEITS et al. 2008; VEITS et al. 2006), ILT herpes virus (LUSCHOW et al. 2001; PAVLOVA et al. 2009a; PAVLOVA et al. 2009b). Several recombinant FP viruses expressing the H5 antigen have been developed (BOYLE et

al. 2000; BUBLOT et al. 2007; HGHIHGHI et al. 2010; KYRIAKIS et al. 2009; MINGXIAO et al. 2006; QIAO et al. 2009; STEENSELS et al. 2009; SWAYNE 2009; SWAYNE and SUAREZ 2000). These vaccines have been reported to be effective in reducing virus shedding and providing clinical protection. However, these vaccines are likely to be used only in birds that are susceptible to infection with the vector virus (CAPUA and MARANGON 2007b). Some studies with these vaccines appear to have shown evidence of a period of growth inhibition in chickens (MINGXIAO et al. 2006). Recently, recombinant ND virus vectors expressing HA of H5 or H7 HPAI virus, developed using reverse genetics, have been licensed for use in China and Mexico (SWAYNE 2009)**.** However, maternal antibodies and active humoral immunity to ND virus vector will interfere with and reduce the protective efficacy. Additionally, biosafety and biosecurity of such live vaccines should be assessed (SWAYNE 2009)**.**

2.8.3 DNA- based vaccine

DNA vaccines have been shown to elicit robust immune responses in various animal species, from mice to nonhuman primates (BARRY and JOHNSTON 1997; LUCKAY et al. 2007). In human trials, these vaccines elicit cellular and humoral immune responses against various infectious agents, including influenza, SARS, SIV and HIV. In addition to their ability to elicit antibody responses, they also stimulate antigen-specific and sustained T cell responses (BARRY and JOHNSTON 1997; GARES et al. 2006; GURUNATHAN et al. 2000; RAVIPRAKASH and PORTER 2006)**.** DNA vaccines have been used in chickens to generate antisera to specific influenza viruses and confer protection against the LP H5N2 strain (KODIHALLI et al. 1997; LEE et al. 2003, 2006). Trials to generate DNA vaccines were done in chickens and mice (BOT et al. 1996; CHERBONNEL et al. 2003; FYNAN et al. 1993; Olsen 2000; PENG et al. 2003; ROBINSON et al. 1997)**.** On the other hand, A single immunization with HA DNA vaccine by electroporation induces early protection against H5N1 AIV challenge in mice (ZHENG et al. 2009)**.** Multivalent HA DNA vaccination protects against highly pathogenic H5N1 AI infection in chickens and mice (RAO et al. 2008)**.** DNA vaccines against AI in poultry have not been as efficacious and consistent as conventional inactivated vaccines.

2.8.4 RNA- based vaccines

To avoid the potential risk of DNA sequence integration into the host genome with DNA vaccination, RNA has been proposed as the expression vector (VIGNUZZI et al. 2001). The mRNA construct encoding the foreign gene delivered intramuscular or

liposome-entrapped mRNA injection. However, antigen expression sufficient to stimulate an adequate immune response is a major problem facing such type of vaccines.

2.8.5 Live vaccine

The use of live influenza vaccines in poultry has never been seriously considered for several reasons. First, there is the possibility of bird-to-bird or farm-to-farm transmission. This may establish AI virus as an endemic infection. Second, the use of live vaccine may cause vaccine-induced respiratory disease in commercial poultry. Third, presumably the most important, there is a potential for recombination with newly introduced AI virus strains to create recombinant AI viruses, which are more pathogenic or has the capability of spreading to different hosts. However, with the advancement in the biotechnology, it may be possible to consider new vaccine approaches using genetically engineered live virus. For example, several experiments showed the possibility of attenuating influenza virus by introducing changes in the PB2 gene, exchanging the promoter region of the NA gene with a different promoter, and generating influenza viruses that have truncated NS1 genes (MURPHY et al. 1997; MUSTER et al. 1991). The study with TK / OR / 71- del, which has truncated NS1 protein, showed attenuation in pathogenicity and a decreased ability to replicate in chickens. The influence of the multi-basic cleavage site (MBS) of the H5 HA on the attenuation, immunogenicity and efficacy of a live attenuated influenza A H5N1 cold-adapted vaccine virus was studied in mice and results showed that restoring the MBS in the H5 HA of the vaccine virus improved its immunogenicity and efficacy, likely as a consequence of increased virus replication, indicating that removal of the MBS had a deleterious effect on the immunogenicity and efficacy of the H5N1 vaccine in mice (SUGUITAN et al. 2009).

2.9 The immune response to influenza infection

To develop vaccines, it is important to understand the immune response against AI virus. The main protective humoral response against AI virus infection is the production of virus-neutralizing antibodies induced by the HA protein and to some extent by the NA protein. In chickens and turkeys, the primary antibody response is initiated by the development of IgM antibody, which can be measured as early as 5 days post-infection. Subsequently, IgY (the mammalian counterpart of IgG) is produced. The mucosal antibody response against AI virus is not clear, but IgA, which is critical for local immunity in respiratory and intestinal tract will likely be

produced after the IgM response similar to what happens after other viral infection in chickens (SUAREZ and SCHULTZ-CHERRY 2000). Local mucosal immunity in avian species is dependent on lymphoid tissue of the head and tracheo-bronchial region. Secretory antibodies against AI virus in the upper respiratory tract are thought to be important for AIV immunity, especially in preventing virus spread since the initial infection usually begins in this area. The protective role of IgA has been shown against several respiratory viruses including ND virus and IB virus (JAYAWARDANE and SPRADBROW 1995; RAJ and JONES 1996). However, similar studies have not been conducted with the mucosal immune response in poultry against AI infection. Neutralizing antibodies, produced against the two surface proteins, HA and NA are the major determinants for a protective immune response. The presence of high titres of humoral antibodies to the HA protein correlates well with protection from clinical disease and with low levels of virus recovery from the trachea of infected birds. For HPAI virus, subtype-specific antibodies prevent viremia, which limits spread from the respiratory or intestinal tract to other susceptible organs such as the kidney and brain. The primary role of the HA protein for inducing protection is further highlighted by the protection of birds with subunit vaccines that contain only the HA protein or the gene that encodes the HA protein. For this reason, vaccination for influenza is targeted primarily toward the HA subtype in poultry. Another surface protein, NA, also elicits neutralizing antibody, and NA specific vaccines can provide some protection against an HPAI challenge in chickens (KAWAOKA and WEBSTER 1988). Although, antibody to the NA protein is thought to be less important than the antibody to the HA protein, the level of reduction in virus shedding after vaccination of mice with NA protein alone was much lower than vaccination with the HA protein. However, greater protection was achieved to mice that were vaccinated with a DNA vaccine that included both the HA and NA genes rather than HA gene alone (CHEN et al. 1998b). Although antibody to the NA protein is valuable for protection, typical killed whole virus vaccines do not induce a good antibody response to the NA protein because much less NA is present in the virion as compared to the HA protein. Detectable antibody responses are also observed against M1, M2, and NP proteins (CHEN et al. 1998b; SLEPUSHKIN et al. 1995). In the mouse model, antibodies to the M2 protein provided some level of protection in terms of preventing virus shedding (SLEPUSHKIN et al. 1995). Although the host usually produces high antibody levels to the M1 and NP proteins, these proteins are not accessible on the surface of the

virion and anti-M1 or NP antibody in the body cannot bind and neutralize the virus. The approach using those influenza genes as a vaccine have attracted the attention of many researches because they are more conserved in amino acid sequence as compared to the HA and NA proteins, and could potentially be a target protein to develop universal or heterosubtypic influenza vaccines (NEIRYNCK et al. 1999). However, vaccines lacking the HA or NA and based solely on conserved internal proteins have not been shown to be protective in poultry. Cellular immunity plays a role in the clearance of the influenza virus (MCMICHAEL et al. 1983). However, reports concerning a role for cell-mediated immunity in protection against AI virus are limited. T cells are the most important cells that mediate the cellular immune response and the T cell subpopulations with diverse functions have been identified in chickens. Infection of BALB/c mice with the H5N9 virus A / Turkey / Ontario / 7732 / 66 resulted in the induction of class-2 MHC-restricted CTL, which recognized an epitope on the H5HA (HIOE and HINSHAW 1989). Similar results were observed with immunization of mice with purified A/Hong Kong / 156/97 H5 recombinant proteins generated in insect cells which induced an H5 –specific CTL response that is primarily class 2-restricted (KATZ et al. 2000). Experiments with a CTL peptide, which consists of an 18 amino acid peptide encompassing the CTL epitope, indicated that a class 2-retricted CTL response, in the absence of HI antibody, was not sufficient to protect mice from death as a result of infection with a highly lethal H5N1virus. Although cellular immunity conferred by H9N2 virus provided some level of protection as measured by mortality rate against subsequent HPAI H5N1infection, all the H9N2 immunized chickens died after challenge with H5N1 influenza virus, but with a longer mean death time and the birds continued to shed virus in their faeces. The cross-protective immunity is based on the presence of large number of killer T cells specific for influenza (SEO et al. 2002). Clearly, much more research should be done in analyzing the immune response to AI virus. Further understanding may lead to more rational design of vaccines to enhance the protective immune response.

2.10 Host response

The ability to cause disease and the ability of the host to respond to influenza varies greatly by species. For example, viruses that are highly pathogenic for chicken show either no disease or only mild disease signs in several different types of ducks (PHILPOTT et al. 1989b). Differences in pathogenicity between species have also been observed using experiential studies with LPAI and HPAI viruses in galliforms

(LAUDERT et al. 1993). For example in a study of two LPAI isolates in chickens and turkeys, the virus was asymptomatic in chicken but caused disease with 25 % mortality in turkeys (LAUDERT et al. 1993). Generally, the differences in disease do not appear to be the result of viruses either being able to infect or not infect a particular species, since evidence of infection occurred with most experimental inoculations of virus. The pathogenesis of AI in different species can also be very different, primarily when comparing ducks to chickens and turkeys. Replication of AI in ducks is believed to be primarily enteric, although respiratory disease has been reported in commercially raised and experientially infected ducks (ALEXANDER et al. 1978). Even when generally characterizing the disease and replication patterns of influenza in ducks caution needs to be used since there are many different species of wild and domestic ducks that may have different response to influenza infection. Differences are also apparent when comparing the immune responses, primarily antibody titres, of different species to AIV infections. Several comparative studies of responsiveness in different species of birds using a variety of antigens suggest that antibody was greater for chicken >> pheasant >> turkey > quail > duck. A similar immunologic response was observed for both vaccination using killed influenza virus or experimental infection with AIV (BRUGH et al., 1979; HOMME and EASTERDAY 1970)**.** Ducks have been reported to develop poor antibody responses and lack HI antibody responses to natural and experimental AI infections (KIDA et al. 1980). The inability of ducks to produce haemagglutinating antibody is probably related to other deficiencies of duck antibody, including precipitation, complement activation, and opsonization. The HA protein has the two main functions of being the virus receptor binding site and containing the fusion domain necessary for the viral RNA to be released into the host cell. The HA protein is glycosylated integral membrane protein that forms a homotrimer on the surface of the virus. At least five antigenic sites have been determined for human influenza viruses, with each site being capable of producing neutralizing antibody (WILEY et al. 1981). Similar observations have also been made for H5 AIV (PHILPOTT et al. 1989b). HA titre in poultry strongly correlates with protection from challenge with virulent viruses of the same HA subtype. The NA protein is an enzymatically active protein that is thought to be important in cleaving sialic acid allowing the virus to be released from the cell surface. Antibody responses are also made to the internal virus proteins, especially the NP and M1 proteins. Both proteins are important antigens when used in

diagnostic tests because both have high sequence conservation that allows the detection of antibody from birds infected by any type A influenza virus. In poultry, the primary methods of detection of type specific antibody are the AGID test and Elisa (MEULEMANS et al. 1987; SNYDER et al. 1985). The mucosal immune response probably also has a role in protection from the HPAI infection because the initial exposure to the virus is through a mucosal surface. However, little direct work has been done with the mucosal immune response in chickens and turkeys.

2.11 Diagnostic tests

2.11.1 Virus isolation and Identification

Basically, there are two approaches for diagnosis of AI: (1) isolation of the virus followed by subtyping using classical methods and (2) molecular characterisation by nucleic acid sequencing. Conventionally, AI virus is isolated by inoculation of swab fluid or tissue homogenates into 9- to 11-day-old embryonated chicken eggs, usually by the chorioallantoic sac route (WOOLCOCK et al. 2001). Depending on the pathotype, the embryos may or may not die within a five-day observation period and usually there are no characteristic lesions to be seen in either the embryo or the allantoic membrane (MUTINELLI et al. 2003). Eggs inoculated with HPAIV-containing material usually die within 48 hours. The presence of a haemagglutinating agent can be detected in harvested allantoic fluid. HA is an insensitive technique requiring at least $10^{6.0}$ particles per ml. If only a low virus concentration is present in the inoculum, up to two further passages in embryonated eggs may be necessary for some LPAIV strains, in order to produce enough viruses to be detected by HA. In the case of HPAIV, a second passage using diluted inoculum may be advantageous for the optimal production of haemagglutination. Haemagglutinating isolates are antigenically characterised by HI tests using (mono-) specific antisera against the 16 H subtypes and, for control, against the different types of avian paramyxoviruses which also display haemagglutinating activities. The NA subtype can be subsequently determined by neuraminidase inhibition (NI) assays, again requiring subtype-specific sera (AYMARD et al. 2003). In case isolates of the H5 or H7 lineages are encountered, their intravenous pathogenicity index (IVPI) needs to be determined to distinguish between LP and HP biotypes (ALLAN et al. 1971). This is achieved by intra-venous inoculation of ten 6-week old chickens with the egg-grown virus isolate. The chickens are observed over a period of ten days for clinical symptoms. Results are integrated into an index which indicates a HPAI virus when values greater than

1.2 are obtained. Alternatively, a HPAI isolate is encountered when at least seven out of ten (75 %) inoculated chickens die within the observation period. The described classical procedures can lead to a diagnosis of HPAI within five days but may demand more than a fortnight to rule out the presence of AIV. In addition, high quality diagnostic tools (SPF eggs, H- and N-subtype specific antisera) and skilled personnel are a prerequisite. Currently, there are no cell culture applications for the isolation of AIV that can achieve the sensitivity of embryonated hen eggs (SEO et al. 2001).

2.11.2 Molecular diagnosis

A more rapid approach, especially when exclusion of infection is demanded, employs molecular techniques, the presence of influenza A specific RNA is detected through the reverse transcription-polymerase chain reaction (RT-PCR) which targets fragments of the M gene, the most highly conserved genome segment of influenza viruses (FOUCHIER et al. 2000), or the nucleocapsid gene (DYBKAER et al. 2004). When a positive result is obtained, RT-PCRs amplifying fragments of the HA gene of subtypes H5 and H7 are run to detect the presence of notifiable AIVs (DYBKAER et al. 2004). When positive again, a molecular diagnosis of the pathotype (LP versus HP) is feasible after sequencing a fragment of the HA gene spanning the endoproteolytic cleavage site. Isolates presenting with multiple basic amino acids are classified as HPAI. Feather pulp was the best sample to detect and isolate HPAIV from infected chicks from 24 hours after inoculation onwards. Kinetic studies on the persistence of virus in infected carcasses revealed that tissues like muscle could potentially transmit infectious viruses for 3 days post-mortem. While other tissues such as skin, feather pulp and brain retained their infectivity as long as 5-6 days post-mortem at environmental temperature (22 - 23 ℃). These results strongly favour feather as a useful sample for HPAIV diagnosis in infected chickens as well as in carcasses (BUSQUETS et al. 2010). PCRs and other DNA techniques have been designed for the detection of Asian lineage H5N1 strains (COLLINS et al. 2002; PAYUNGPORN et al. 2004). Non-H5/H7 subtypes can be identified by a canonical RT-PCR and subsequent sequence analysis of the HA2 subunit (PHIPPS et al. 2004). There are also specific primers for each NA subtype. A full characterisation might be achievable within three days, especially when real time PCR techniques are used. An exclusion diagnosis is possible within a single working day. The disadvantages of molecular diagnostics are economic issues, although, if available, many samples can be analysed by less personnel in grossly shorter times in

comparison to virus isolation in eggs. However, it should not be kept secret that each PCR or hybridisation reaction, in contrast to virus isolation in eggs, harbours an intrinsic uncertainty related to the presence of specific mutations in a given isolate at the binding sites of primers and/or probes which might render the assay false negative. Thus, a combination of molecular (e.g. for screening purposes) and classical methods (e.g. for final characterisation of isolates and confirmation of diagnosis of an index case) may help to counterbalance the disadvantages of the two principles. Rapid assays have been designed for the detection of viral antigen in tissue impression smears and cryostat sections by use of IF or by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) and dip-stick lateral flow systems in swab fluids. So far, these techniques have been less sensitive than either virus isolation or PCR, and therefore might be difficult to approve for a legally binding diagnosis, especially of an index case (CATTOLI et al. 2004; DAVISON et al. 1998; SELLECK et al. 2003). The use of pen side tests in the veterinary field is still in its infancy and needs further development.

2.11.3 Serological diagnosis

Group-specific antibodies (influenza virus type A) against the NP can also be detected by AGID and by ELISA (JIN et al. 2004; MEULEMANS et al. 1987). Various forms of enzyme immunoassays have recently been developed and prove to be more sensitive and specific than AGID and HI. The AGID test requires large quantities of reagents and 24-48 hours for results to be obtained. Furthermore, the AGID test may not be suitable as a universal assay for some other species of birds; serum samples from water fowl do not contain good precipitating antibodies. The HI test is more sensitive and rapid than the AGID test, but it is complicated due to the existence of 16 HA subtypes of AIV.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Avian influenza virus

A highly pathogenic influenza A subtype H5N1 (A / Thailand / 1 (Kan-1) / 2004) isolated from Thailand (PUTHAVATHANA et al. 2005) was obtained from Dr. Puthavathana, Department of Microbiology, Bangkok as a supernatant of infected MDCK-cells. The published sequence of this virus (Accession number: AY555150) was used for isolation of coding fragments of epitope based truncated sequences of HA. Also, this virus was used for preparation of inactivated H5N1 antigen to be used for vaccination and in serological tests.

3.1.2. Bacteria

XL10-Gold® ultracompetent cells (Stratagene, La Jolla, CA, USA) were used. These cells exhibit the Hte phenotype, which increases the transformation efficiency of ligated and large DNA molecules. The genotype of XL10-Gold ultracompetent® cells as follow: Tetr Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*q*Z*Δ*M15* Tn*10* (Tetr) Amy Camr].

3.1.3 *Pichia pastoris* strains

Two *P. pastoris* strains, supplied by Invitrogen Life Technologies (Invitrogen, Karlsruhe, Germany) were used. GS115 (*his4*) strain is an auxotrophic mutant deficient in histidine dehydrogenase, while SMD1168 H *(his4, pep4*) is additionally defective in the vacuole peptidase *A (pep4)*.

3.1.4 Expression vector

For recombinant protein expression, pGAPZαC vector (Invitrogen GmbH, Karlsruhe, Germany) was modified and designated pAOX. Briefly, the AOX promoter was amplified from SMD1168H genomic DNA with appropriate primers and used to replace the GAP promoter. 5´ AOX1 promoter region induces expression in the presence of methanol. The plasmid contains on α-factor signal sequence (responsible for secretion of target protein), multiple cloning sites (MCS) (to insert gene of interest) and polyhistidine (6xHis-tag to facilitate purification and protein detection). Moreover, Zeocin® *She ble* resistance gene is incorporated into the cloning vector and used as a selectable marker for transformation (Figure 1).

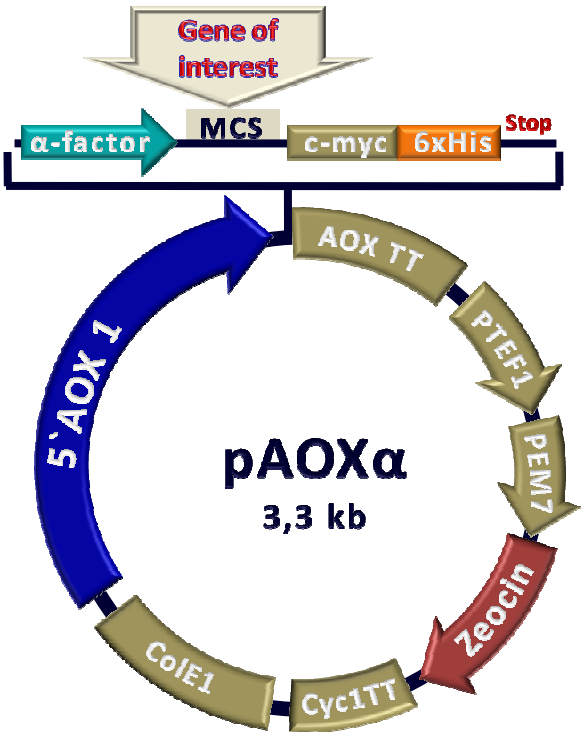


Figure (2): Schematic diagram of pAOX vector (modified pGAPZαC vector, Invitrogen)

3.1.5 Cells

Chicken red blood cells (RBCs) suspension

Blood was collected from wing vein of chickens in sterile tubes containing 3.8 % sodium citrate solution. Equal volume of PBS was added and the erythrocytes were centrifuged at 1000 rpm for 10 min. After 3 times wash cycles, 1 % RBCs in PBS was used for haemagglutination and HI tests.

Vero cells

The Vero cells CCL 81, derived from kidney epithelial cells of the African Green Monkey were used for influenza virus propagation, virus titration and in micro-neutralization test (µNT).

3.1.6 Animals

Mice

Eight BALB/c Mice were used to study the immunogenicity of different recombinant polypeptides (P1, P2 and rHA1).

Chickens

Twenty- four inbred commercial layer chickens were purchased from Lohman Animal Health (Cuxhaven, Germany). Chickens had a history of immunization against salmonellosis, infectious bronchitis (IB), Newcastle disease (ND), infectious bursal

disease (IBD), avian encephalomyelitis (AE), and ILT. History of vaccination program was shown in the following table:

Age	Vaccination
1-day	Salmonellosis
2 weeks	IB
3 weeks	ND
4 weeks	IBD
5 weeks	IB
6 weeks	ND
7 weeks	Salmonellosis
9 weeks	ILT
11 weeks	AE
13 weeks	IB
14 weeks	ND
15 weeks	Salmonellosis

3.1.7 Positive and negative serum samples

Positive chicken sera

One hundred and seventy nine serum samples were obtained from Egyptian broiler chickens, previously vaccinated once with commercial inactivated H5N2 (A / chicken / Mexico / 232 / 94 / CPA) vaccine at 7-day-old. These chickens were vaccinated also against IBD, ND, Marek´s disease (MD) and IBV. Blood samples were collected from wing vein or by slaughtering at 4 weeks post vaccination and kept in a slope position at 37 ^{o}C for one hour then at 4 ^{o}C overnight. Sera were then separated by centrifugation at 3000 rpm for 10 minutes and stored at -20 ^{o}C till tested.

Negative chicken sera

25 serum samples obtained from influenza non-vaccinated broiler chickens and tested negative by AGID, HI (using H5N1 Thailand antigens) and Western blot were used as negative control in Elisa. These chickens were vaccinated against IBD, ND, MD and IBV.

Duck sera

Five serum samples were obtained from vaccinated Egyptian duck, aged 20 weeks. Ducks were vaccinated 3 times with commercial H5N2 vaccine. Also, three negative serum samples were obtained from non vaccinated duck as a negative control. Ducks have a history of vaccination against duck virus enteritis (DVE) and duck virus hepatitis (DVH). These serum samples were analyzed by rHA1-Elisa and HI test.

3.1.8 Media for bacterial and yeast cultures

3.1.8.1 Media for bacterial culture

Low salt LB broth medium (LS-LB)

1 % (w/v)	Peptone (from casein)
0.5 % (w/v)	Sodium chloride
0.5 % (w/v)	Yeast extract

Peptone, NaCl, and yeast extract were dissolved in dH_2O; pH was adjusted with NaOH to 7.5. After autoclaving, the medium was left to cool and zeocin® was added at a concentration of 25 µg / ml.

Low salt LB agar medium (LS-LB agar)

1 % (w/v)	Peptone (from casein)
0.5 % (w/v)	NaCl
0.5 % (w/v)	Yeast extract
1.5 % (w/v)	Agar- agar

Peptone, NaCl, yeast extract and agar were dissolved in dH_2O, after autoclaving, the medium was left to cool and zeocin® was added at a concentration of 25 µg / ml.

3.1.8.2 Media for yeast

Yeast peptone dextrose (YPD)

1 % (w/v)	Yeast extract
2 % (w/v)	Peptone (from casein)
2 % (w/v)	Dextrose (D-Glucose)

Yeast extract and peptone were dissolved in 900 ml dH_2O. After autoclaving 100 ml 20 % (w/v) sterile glucose solution were added.

Yeast peptone dextrose agar (YPD-agar)

1 % (w/v)	Yeast extract
2 % (w/v)	Peptone
2 % (w/v)	Dextrose (D-Glucose)
2 % (w/v)	Agar agar

Yeast extract, peptone and agar were dissolved in 900 ml dH_2O. After autoclaving 100 ml 20 % (w/v) glucose solution were added.

Yeast peptone glycerol medium (YP-Gly)

1 % (w/v)	Yeast extract
2 % (w/v)	Peptone
1% (v/v)	Glycerol

Yeast extract and peptone were dissolved in dH_2O. After autoclaving 2 % (v/v) glycerol were added just before use.

Yeast peptone methanol, pH 8 (YP MeOH, pH8)

1 % (w/v)	Yeast extract
2 % (w/v)	Peptone
60 mM	Tris- HCl (pH 8.3)
1% (v/v)	Methanol

Yeast extract and peptone were dissolved in dH2O. After autoclaving, sterile tris-Cl solution, pH 8.3 was added. Methanol was added just before use.

Yeast peptone methanol, pH 6 (YP MeOH, pH 6)

1 % (w/v)	Yeast extract
2 % (w/v)	Peptone
1.34 % (w/v)	Yeast nitrogen base
100 mM	Potassium dihydrogen phosphate (KH_2PO_4, pH 6)
1 % (v/v)	Methanol

Yeast extract, peptone and yeast nitrogen base were dissolved in dH_2O. After autoclaving, sterile KH_2PO_4 (pH 6) was added. Methanol was added just before use.

3.1.9 Buffers and solutions

3.1.9.1 Buffers and solutions for *E-coli* transformation

Tfb1

300 mM	Potassium acetate
100 mM	Rubidium chloride (RbCl)
10 mM	$CaCl_2$
50 mM	$MnCl_2$

Dissolved in 300 ml dH_2O and pH was adjusted with 10 % acetic acid to 5.8. 75 ml glycerine 99 % was added. The mixture was completed to 500 ml with dH_2O and filtered with 0.2 µm bacteriological filter. This buffer was used for preparation of competent *E-coli* cells.

Tfb2

10 mM	Potassium acetate
75 mM	RbCl
10 mM	$CaCl_2$

Dissolved in 50 ml dH_2O and pH was adjusted with 1 M KOH to 6.5. 15 ml glycerine was added. The mixture was completed to 500 ml with dH_2O and filtered with 0.2 µm bacteriological filter. This buffer was used for preparation of competent *E-coli* cells.

3.1.9.2 Buffers and solutions for DNA and protein analysis

Anode buffer

25 mM	Tris- Cl

Dissolved in dH_2O, pH was adjusted to 8.9 with HCl

Block buffer for Western blot

4 % (w/v)	Skim milk in 1 x PBS

5x Cathod buffer

100 M	Tris-Cl
0.5 M	Tricine
1.73 mM	Sodium dodecyl sulfate (SDS)

Gel drying solution

30 %	Ethanol (96 %)
10 %	Glycerine

This solution was used for drying of the silver stained gel (SDS-PAGE) to be used as documentation.

10x PBS

1.37 M	NaCl
27 mM	Potassium chloride
80 mM	Di-sodium hydrogenphosphate
18 mM	Potassium di-hydrogen phosphate

Dissolved in dH_2O and pH was adjusted to 7.4 with NaOH or HCl.

50x TAE buffer

2 M	Tris
5.71 % (v/v)	Acetic acid
100 mM	Ethylenediamintetraacetic acid (EDTA)

Dissolved in dH_2O and pH was adjusted to 8.5 with NaOH

TNE Buffer

10 mM	Tris-Cl
100 mM	NaCl
1mM	Ethylenediamintetraacetic acid (EDTA)

Dissolved in dH_2O and pH was adjusted to 7.4 with NaOH or HCl

Transfer buffer

25 mM	Tris
192 mM	Glycerine
20 % (v/v)	Methanol
in dH_2O	

2x Tricine sample buffer

5 % (w/v)	SDS
12.5 % (v/v)	1 M Tris-HCl pH, 6.8
20 % (v/v)	Glycerine
0.08 % (w/v)	Bromophenol blue
in dH_2O	

3.9.3 Buffers and solutions for polypeptide purification

Elution buffer (1)

100 -300 mM	Imidazole
300 mM	NaCl
50 mM	Tris- Cl

Dissolved in dH_2O and pH was adjusted to 7 with NaOH or HCl

Elution buffer (2)

300 mM	NaCl
50 mM	Tris- Cl

Dissolved in dH_2O and pH was adjusted to 2-3 with HCl

Elution buffer (3)

150 mM	NaCl
10 mM	PBS
0.4 M	methyl α- D- manopyranoside

Dissolved in dH_2O and pH was adjusted to 7.4 with NaOH or HCl

Wash buffer (1)

10 mM	Imidazole

300 mM	NaCl
50 mM	Tris- Cl

Dissolved in dH_2O and pH was adjusted to 7 with NaOH or HCl

Wash buffer (2)

300 mM	NaCl
50 mM	Tris- Cl

Dissolved in dH_2O and pH was adjusted to 6.5 with NaOH or HCl

Wash buffer (3)

150 mM	NaCl
10 mM	PBS

Dissolved in dH_2O and pH was adjusted to 7.4 with NaOH or HCl

3.1.9.4 Buffers and solutions for Elisa

Block buffer

1x	PBS
3 %	Bovine serum albumin (BSA)
0.05 %	Tween20

Coating buffer

200 mM	Sodium bicarbonate

Dissolved in dH_2O and pH was adjusted to 7.4 with NaOH or HCl

Substrate

Tetramethylbenzidin (TMB) stock solution

1 mg TMB (w/v) dissolved in 1 ml Dimethyle Sulfoxide solution (DMSO) and stored at – 20 until used.

Sodium acetate citrate buffer

0.1 M	Sodium acetate
500 ml	dH_2O

pH was adjusted with 0.1 M citric acid monohydrate to 6 and stored at – 20 until used

Working substrate

1 ml	TMB stock solution
9 ml	Sodium acetate citrate buffer
2 µl	H_2O_2

Wash buffer

1X	PBS
0.05 %	Tween20

In Millipore water

3.1.10 Reagents for molecular biology

Reagent	Supplier
Ethidium bromide	Sigma, Germany
dNTP-solution	Fermentas, Leon-Rot, Germany
10x Denaturating buffer	New England Biolabs, UK
10 GS buffer	New England Biolabs, UK
Hi-Di-Formamide	Applied Biosystem
HPLC- water	Roth, Karlsruhe, Germany
Magnesium chloride solution	Invitrogen life Technology, Karlsruhe, Germany
Magnesium sulfate solution	Fermentas, Leon-Rot, Germany
Ni-NTA	Quiagen, Helden, Germany
Pfu 10x Buffer	Fermentas, Leon-Rot, Germany
Polyethylene glycol solution 4000	Fermentas, Leon-Rot, Germany
6X Loading dye	Fermentas, Leon-Rot, Germany
Lectin peroxidise concanavalin	Sigma, Germany
Tunicamycin	Sigma, Munich, Germany
Zeocin	Invitrogen life Technologies, Karlsruhe

3.1.11 Enzymes

Enzyme	Supplier
Restriction enzymes	Fermentas, Leon-Rot, Germany
Endoglycosidase	New England Biolabs, UK
Pfu polymerase	Fermentas, Leon-Rot, Germany
Plantium- Taq polymerase	Invitrogen life technologies
T4-Ligase	Fermentas, Leon-Rot, Germany

3.1.12 Protease Inhibitors

Pepstatin	Sigma Aldrich, Munich, Germany
Phenylmethylsalfonylfluorid (PMSF)	Roth, Karlsruhe, Germany

3.1.13 Standards

Gene Ruler 1 kb DNA Ladder	Fermentas, Leon-Rot, Germany
Gene Ruler 100 bp DN A – Ladder	Fermentas, Leon-Rot, Germany
SDS- PAGE protein marker	Serva, Heidelberg, Germany

3.1.14 Antibodies

Antibody	Supplier
FITC monoclonal anti-chickens	Fermentas, Leon-Rot, Germany
FITC monoclonal anti-maus	Fermentas, Leon-Rot, Germany
Goat anti-bird IgG HRP	Biomol, Hamburg, Germany
Influenza anti-nucleoprotein	Gift from Prof. Dr. Christian Gassoy
Maus anti Histag	Dianova, Hamburg, Germany
Polyclonal rabbit anti-maus IgG HRP	Dako, Hamburg, Germany
Rabbit anti-chicken IgY HRP conjugate	Millipore, Schwalbach, Germany

3.1.15 Reagents for cell culture

Reagent	Supplier
Crystal violet	Sigma, Germany
Dulbecco's modified eagle media (DMEM + GlutaMAX ^{TM}-1)	Gibco Invitrogen Corporation
Dimethyle sulfoxide solution (DMSO)	Sigma, Germany
Fetal calf serum (heat inactivated)	Biochrom, Berlin, Germany
PBS 10x (-$Ca^{2+,}$ Mg^{2+})	Gibco Invitrogen Corporation
Penicillin	Jenapharm, Jena, Germany
Streptomycin	Sanavita, Germany
Trypan blue solution (0.4 %)	Gibco Invitrogen Corporation
Trypsin	Gibco Invitrogen Corporation

3.1.16 Kits

Kit	Supplier
Big dye terminator cycle sequencing kits	Applied Biosystem, USA
Biorad protein assay	Biorad, Hercules, CA, USA
Pichia easyComp^{TM} Kit	Invitrogen life technologies,
Wizzard SV® g el and PCR clean-up system	Promega, Mannheim, Germany
Flock check commercial Elisa kits	Synbiotic, USA
ZipTip® pipette tip	Millipore, Schwalbach, Germany

3.1.17 Adjuvant

Gerbu Adjuvant 10	Gerbu Biotechnik GmbH, Gaiberg, Germany.

3.1.18 Fine chemicals

Chemical	Supplier
Ammonium sulfate	Sigma ,Germany
Aceton	Roth, Karlsruhe
Acetic acid	Roth, Karlsruhe
Acrylamide	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Agar agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
APS	Applichem, Darmstadt
Bromophenol	Fluka, Swizerland
BSA	Sigma, Germany
$CaCl_2$	Roth, Karlsruhe
Citric acid	Roth, Karlsruhe
DEPC	Fluka, Swizerland
D-Glucose	Applichem, Darmstadt
D-Sorbitol	Applichem, Darmstadt
3,3´-Diaminobenzidin tetrachloride	Applichem, Darmstadt
Disodium hydrogen phosphate	Applichem, Darmstadt
EDTA	Roth, Karlsruhe
Ethanol	Roth, Karlsruhe
Formaldehyde	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
HCl	
Magnesium sulfate ($MgSo_4$)	Roth, Karlsruhe
$MnCl_2$	Merck, Darmstadt
Peptone (from casein)	Applichem, Darmstadt
Polyethylene glycol 1500	Roth, Karlsruhe
Potassium acetate	Roth, Karlsruhe
Potassium chloride	Applichem, Darmstadt
Potasium dihydrogen phosphate	Applichem, Darmstadt
Potassium sulfate	Roth, Karlsruhe
Rubidium chloride	Fluka

SDS	Roth, Karlsruhe
Silver nitrate	Vitalia GmbH, Sauerlach
Skim milk	Applichem, Darmstadt
Sodium acetate	Roth, Karlsruhe
Sodium carbonate	Applichem, Darmstadt
Sodium chloride	Roth, Karlsruhe
Sodium hydroxide	Roth, Karlsruhe
Sodium thiosulfate – hydrate	Roth, Karlsruhe
Sulfuric acid	Roth, Karlsruhe
Sulfuric acid 0.5 M (Elisa stop sol.)	Roth, Karlsruhe
Tetramethyl-ethylendiamine (TEMED)	Applichem, Darmstadt, Germany
Tetramethylbenzidin (TMB)	Serva, Heidelberg
Trichloroacetic acid	Roth, Karlsruhe
Tris base	Roth, Karlsruhe
Tris-HCl	Applichem, Darmstadt
Tween 20	Applichem, Darmstadt
Urea	Roth, Karlsruhe
Yeast extract	Roth, Karlsruhe
Yeast nitrogen base	Fluka, Steiheim

3.1.19 Instruments and measurements

Instruments and measurements	**Supplier**
ABIPRISM™ 310 genetic analyzer	Applied Biosystem, USA
Autoclave	Münchner Medizin Mechanik GmbH, Germany
Balance - BP2100 S - BP 211 D	Sartorius, Göttingen, Germany
Biostat C plus-C15-3 fermenter	Sartorius BBI System GmbH, Melsungen, Germany
Block cycler - PTC 200 DNA Engine® thermal cycler	Biorad, München, Germany
Blotting apparatus (Mini Trans-Blot®)	Biorad, München, Germany

Centrifuge - Eppindorf centrifuge 5417 R - Labor centrifuge 1K15 - Rotina 46R - Vacuum centrifuge speed vac - Sorvall ® Ultraspeed centrifuge	 Eppindorf; Hamburg, Germany Sigma, Germany Hettich, Tuttlingen, Germany Savant Kendro, US
Electrophoresis Agarose gel electrophoresis - Mini Sub® Cell GT - Wide Mini® Sub Cell GT - Electrophoresis chamber for polyacrylamide gel Power supply apparatus - Power Pac 300 - Power Pac 3000	Biorad
Gel documentation system Multimage™ Light Cabinet	 Alpha Innotech Corporation
Heating block	Eppendorf, Hamburg, Germany
Ice machine	Scotsman, USA
Incubators - Kelvitron®t - Incubator for yeast - Steri-Cult-HEPA filtered IR cell culture	 Heraeus, Hanau, Germany WTB Binder , Tuttlingen, Germany Forma Scientific
Laminar Flow cabinet	Heraeus Instruments
Microwave (Panasonic Pro II 1400)	Masushita electric industrial Co
Microscopes - Fluorescent microscope DM IRB (inverse) - Fluorescent microscope DMRA - Fluorescent microscope DMRB - Inverse stereomicroscope DMIL	 Leica, Germany
Refrigerators and freezers -80 ^{o}C	Liebherr, Ochsenhausen Thermo Scientific
pH-Meter pH 540 GLP	Wissenschaftlich- Technische

	Werkstätten, Germany
Thermo mixer compact	Eppendorf, Hamburg, Germany
Photo spectrometer NanoDrop® SmartSpec™ 3000	 peQLab Biotechnologie Gmbh Biorad, München, Germany
Vortexer (Vortex Genie 2)	Scientific Industries
Water bath	GFL, Burgwedel, Germany

3.1.20 Consumption materials

Materials	**Supplier**
Cell culture plate (96, 24, 8 wells)	Greiner Bio one, Frickenhausen
Cover slides	Braunschweig, Germany
Cryo tubes	Greiner Bio-One, Frickenhausen,
Disposable canula syringe	Braunschweig, Germany
Elisa plates (microlon high binding)	Greiner Bio- One, Frickenhausen,
Falcon tubes	Bedford, USA
Filter tip gilson (10, 20, 200, 1000 µl)	Greiner Bio-One, Frickenhausen
Micro tube 2 ml with cap	Sarstedt, Nümbrecht
Nitro-cellulose membrane	Millipore, Schwalbach, Germany
Parafilm	Pechiney plastic Packaging, Chicago
Petri dish	Greiner Bio-One, Frickenhausen
Pipette tips	Greiner Bio one, Frickenhausen
	Sartedt
Plastic pipette	VWR International, USA
Reactions containers (50, 15,1.5)	Roth, Karlsruhe
Rotilab® Spritzenfilter, 0.45 µm	Roth, Karlsruhe
Rotilab® Spritzenfilter, 0.2 µm	Roth, Karlsruhe
Rotilab® Micro titre plates (U- shape)	Roth, Karlsruhe
Rotilab® Micro titre plates (V- shape)	Roth, Karlsruhe
Slides 76 X 26	Greiner Bio-One, Frickenhausen
Tissue culture plastic flasks	Sartorius-Stedim, Biotech GmbH,
Vivaspin20	Göttingen, Germany

3.2 Methods

3.2.1 Expression of truncated sequences of influenza subtype H5 in *P. pastoris*

3.2.1.1 Amplification of truncated sequences

To identify regions within H5 protein that are highly conserved, protein alignments were performed using MacVector™ 7.0. Four non-overlapping sequences of different functional domains of influenza A virus subtype H5 were chosen and designated P1, P2, P5 and rHA1 (the criteria of these epitopes are described in table 1, and appendix)**.** RNA was extracted and cDNA of influenza A subtype H5N1 (A / Thailand / 1 (Kan-1) / 2004) was synthesized. PCR was used to amplify 4 coding DNA fragments of epitope based truncated sequences of HA. Two primers for each coding sequence were used for a series of synthetic reactions. The primers were designed with specific restriction enzymes sites to create compatible ends (vector-PCR products). In the reverse primers, stop codons were not induced in order to fuse the coding sequence with 6xHis-tag (Table 1). PCR reaction was done in a 50 µl final volume containing 5 µl 10x polymerase buffer, 1.25 µl dNTP (10 mM), 1 µl of each primer (10 µM), 0.5 µl DNA polymerase (2.5 U / µl), 1 µl DNA template (200 ng / µl) and 10.25 µl deionised water. The block cycler (PTC 200 DNA Engine® Thermal Cycler) program was as follows: 95 ^{o}C, 3´; 5 X (95 ^{o}C, 3´; 55 ^{o}C, 20´´; 72 ^{o}C / 90´´; 29X (95 ^{o}C, 30´´; 70 ^{o}C, 20´´; 72 ^{o}C, 90´´); 72 ^{o}C, 90´´

3.2.1.2 Agarose gel electrophoresis

Agarose powder was added to 1X TAE Buffer to a final concentration of 0.8 % (w/v). The slurry was heated in a microwave oven until the agarose was dissolved. 5 µl ethidium bromide were added after cooling (70 ^{o}C) and the agarose solution was poured into the mold and the comb was properly positioned. After the gel was completely set, it was transferred into electrophoresis tank and covered with 1 X TAE Buffer, DNA samples were mixed with 6x DNA loading dye (5 vol. DNA solution plus1 vol. DNA loading dye) and loaded into the slots of the gel. As a size standard, 50 bp, 100 bp and 1Kb GeneRuler DNA Ladder™ were used. The gel was run in TAE buffer under the voltage of 120 V for 30 minutes at RT. The gel was visualized by ultraviolet light and documented by MultiImage™ light cabinet with ChemiImager 4000 computer program.

Table (1): Primers and peptide description

[a]Peptide	[b]Primer sequence	[c]Peptide description
P1	**Site E-sense:** GTA **CTC GAG** AAG AGA GAG GCT GAA GCA GAT CTA GAT GGA GTG AAG CC **Site E-as:** CAT **GCG GCC GC**C TTC TCC ACT ATG TAG GAC C	corresponds to the neutralizing epitope of site E of H3 (WILEY et al. 1981), conserved in H5, 40 amino acids residues long, molecular mass is 7.5 kDa.
P2	**RBS-sense:** GTA **CTC GAG** AAG AGA GAG GCT GAA GCA AAT AAT ACC AAC CAA GAA GAT C **RBS-as:** CAT **GCG GCC GC**G TCC CCT TTC TTG ACA ATT TTG	consists of the receptor binding site, site D and parts of site B, conserved in H5, 97 amino acid residues long, contains a glycosylation site, molecular mass is 13.9 kDa
P5	**Site A-sense:** ´5 GTA **CTC GAG** AAG AGA GAG GCT GAA GCA TCA TTA GGG GTG AGC TCA GC 3´ **Site A-as**: 5´CAT **GCG GCC GC**G TAT GTA CTG TTC TTT TTG ATA AGC C 3´	conformational epitope in H5 (PHILPOTT et al. 1989a), not conserved in H5, 30 amino acid residues long, contains a glycosylation site, molecular mass is 6.5 kDa
rHA1	**HA1-sense:** 5´GTA **CTC GAG** AAG AGA GAG GCT GAA GCA GAT CAG ATT TGC ATT GGT TAC C 3´ **HA1-as:** ´5GAT **GCG GCC GC**T CTT TGA GGG CTA TTT CTG AGC C´3	contains the majority of those antigenic determinants of HA that are responsible for generation of virus-neutralizing antibodies, 320 amino acid residues long, contains 5 glycolsyation sites, molecular mass is 39.6 kDa

[a]P1, P2, P5 and rHA1 coding DNA fragments of epitope based truncated sequences of HA influenza A subtype H5N1 (A / Thailand / 1 (Kan-1) / 2004).

[b]Bold sequences indicate the sequence of restriction sites (Xho1 in sense primers and Not1 in antisense primers) , the sequence of alpha factor is underline.

[c]Molecular mass calculated for his-tag polypeptide when alpha factor is completely processed (secreted in supernatant).

3.2.1.3 DNA purification

PCR product was purified using Wizard SV® Gel and PCR Clean-Up system according to manufacturer. The Wizard® SV Gel and PCR Clean-Up kit is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. Briefly, one SV Minicolumn® was placed in a collection tube for each PCR reaction.

PCR product was placed to the SV Minicolumn® assembly and incubated for 1 minute at room temperature, followed by centrifugation at 16,000 ×*g* (14,000 rpm) for 1 minute. The liquid in the collection tube was discarded and 700 µl of membrane wash solution was added, previously diluted with 95 % ethanol, followed by centrifugation for 1 minute at 16,000 ×*g* (14,000 rpm). Again, the liquid in the collection tube was discarded and 500 µl of membrane wash solution was added followed by centrifugation for 5 minutes at 16,000 ×*g*. The column assembly was re-centrifuged for 1 minute with the micro-centrifuge lid open after empting the collection tube. Carefully SV Minicolumn® was transferred to a clean 1.5 ml micro-centrifuge tube and 50 µl of nuclease-free water (supplied with the kit) were added directly to the centre of the column without touching the membrane with the pipette tip, incubated at room temperature for 1 minute and centrifuged for 1 minute at 16,000 ×*g.* The eluted DNA was stored at 4 ℃ or at -20 ℃.

3.2.1.4 Cloning of PCR product

Both DNA product and pAOX plasmid were digested by Xho1 and Not1 restriction enzymes. The reaction composition was shown as follows: 5.5 µl O-buffer, 50 µl purified DNA, 1 µl Xho1 and 1 µl Not1 restriction enzymes. The reaction mixture was incubated 37 ℃ for 2 hrs. DNA was purified again using Wizard SV® Gel and PCR Clean-Up system as described before.

3.2.1.5 DNA ligation

DNA concentration was determined using NanoDrop®. The method is based on the spectrophotometric measurement of the absorption. The principle of NanoDrop® depends on the measurement of the absorbance at A260. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as “pure”. Digested P1, P2, P5 and rHA1 DNA with Xho1 and Not1 were mixed with digested pAOX vector at a ratio of 1:6 in a 10 µl of ligation reaction containing 1 µl T4-DNA ligase (2U), 1 µl 10x ligation buffer, and 1 µl 50 % (w/v) polyethylene glycol (PEG) 4000 (supplied with the enzyme). The mixture was incubated at room temperature for 2 hrs.

3.2.1.6 Preparation of competent *E-coli*

Firstly, a fresh plate of cells was prepared by streaking out cells from a frozen stock and growing at 37 ^{o}C. An individual colony was set in 5 ml low salt LB – medium (LS-LB) without antibiotic. 1 ml of overnight culture was inoculated in a flask containing 100 ml LS-LB medium. The flask was incubated at 37 ^{o}C with aeration until the

culture reached an OD_{595} of 0.200 - 0.300 (approximately 2 hrs). Culture was incubated on ice for 5 minutes then transferred to two 50 ml falcon tubes and centrifuged 2500 rpm for 10 minutes at 4 ^{o}C. Pellets were gently resuspended in 20 ml Tfb I (for each tube) and centrifuged 3500 rpm for 10 minutes at 4 ^{o}C. The pellet was resuspended in 4 ml Tfb II and incubated on ice for 15 minutes. Competent cells were divided into convenient aliquots with equal amount of glycerine and frozen at – 80 ^{o}C.

3.2.1.7 Transformation of competent *E-coli*

For amplification of plasmid construct, XL10-Gold® ultracompetent *E- coli* was used. Five microlitres of ligation mixture was mixed by stirring gently with pipette tip into competent *E. coli* cells which thawed on ice just before transformation. The mixture was placed on ice for 30 minutes and incubated at 42 ^{o}C for 30 seconds then rapidly placed on ice for 2 minutes. Three hundred microlitres of LB liquid medium was added before shaking at 37 ^{o}C for 1 hour. Transformed cells were spread on LB agar plate containing 25 µg / ml Zeocin™ (100 µl / plate). Plates were incubated overnight at 37 ^{o}C.

3.2.1.8 Plasmid isolation from *E-coli*

Plasmid isolation was done by Zyppy™ Plasmid Miniprep kit according to the manufacturer. Plasmid DNA isolated by this method suited for ligation, sequencing and restriction digestion. It is a modified alkaline lysis method. All buffers were supplied by the manufacturer. Briefly, selected colonies were picked up and inoculated into 5 ml LS- LB liquid medium pH, 7.5 and incubated overnight at 37 ℃. 600 µl of bacterial culture grown in LB medium were added to a 1.5 ml micro-centrifuge tube. 100 µl of 7X lysis buffer (blue) were added and mixed by inverting the tube 4-6 times. 350 µl of cold neutralization buffer (yellow) were added and mixed thoroughly. The sample was inverted an additional 2-3 times to ensure complete neutralization, followed by centrifugation at 11,000 – 16,000 xg for 2-4 minutes. The supernatant (~900 µl) was transferred into the provided Zypy-Spin™ IIN column. The column was placed into a collection tube and centrifuged at 16,000 xg for 15 seconds. The flow-through was discarded and 200 µl of Endo-wash buffer were added to the column followed by centrifugation at 16,000 xg for 15 seconds. 400 µl of Zyppy-Spin™ wash buffer were added to the column and centrifuged at 16,000 xg for 30 seconds. The column was transferred into a clean 1.5 ml micro-centrifuge tube then 30 µl of Zyppy™ elution buffer were added directly to the column matrix and let

stand for one minute at room temperature, followed by centrifugation at 16,000 xg for 15 seconds to elute the plasmid DNA.

3.2.1.9 Analysis of plasmids

The recombinant plasmids (designated pAOX H5-P1, pAOX H5-P2, pAOX H5-P5 and pAOX H5-HA1) were analysed by restriction digestion and sequencing. pAOX H5-P1, pAOX H5-P2 and pAOX H5-HA1 plasmids were subjected to double digestion with Bgl II, whereas Bg1 II and Xho1 were used for double digestion of pAOX H5-P5.

3.2.1.10 Sequencing of the insert

For this purpose, the plasmid was isolated from *E- coli* and its concentration was identified using NanoDrop®. PCR was performed using PGAPZ seq 1 primer (5´-GTCCCTATTTCAATCAATTGAA-3´). The sequencing reaction consisted of 4 µl of dye-terminator reaction mix, 2 µl of 10 µM sequencing primer, 2 µl HPLC- H_2O and 200-500 ng (in 2 µl) of plasmid. The cycle sequencing reaction was performed by using the following thermal cycler program**:** 96 ℃, 2´; 25 x (96 ℃ ,10´´; 55 ℃ , 5´´ ; 60 ℃, 4´); 60 ℃, 4´. The PCR product was then pre cipitated by ethanol. 70 µl HPLC water and 10 µl 3 mM Na - acetate (pH 4.6-4.8) were added to 10 µl of the sequencing reaction in 1.5 ml ependorf. After addition of 250 µl 100 % ethanol, the sample was vortexed and centrifuged 14000 rpm / 35 min at 22 ℃. Supernatant was discarded and 250 µl of 70 % ethanol was added followed by centrifugation 14000 rpm / 35 min at 22 ℃. Pellet was dried in speed va cuum for 10 min. The pellet was resuspended in 20 µl Hi- Di-Formamide. Sequencing the gene of interest was analyzed in an ABIPRISM™ 310 genetic analyzer.

3.2.1.11 *P. pastoris* transformation

P. pastoris competent cells were prepared according to Invitrogen manual and stored frozen until used. Native cells were prepared as follow: 50 ml YPD medium were inoculated with stationary culture (24 hrs) of native yeast cells grown in YPD medium to OD_{600}= 0.02- 0.03. Cells were incubated for 18 – 20 hrs at 30 ℃ (250 rpm) until OD_{600} = 15-30. Aliquots were prepared (500 µl – 3-7x 10^8) cells) and stored at – 20 ℃ until use. The shelf life is for several months. Transformation was done according to EasyComp™ kits after some modifications described by Dr Kathrin Rall (Institute of Virology, Faculty of Medicine, Leipzig University, Germany). Briefly, after analysis of the insert, an amount of recombinant plasmid DNA was prepared. 5-10 µg (per transformation) were linearized by Bstx1, and dried in a vacuum centrifuge. One aliquot of competent cells (for one transformation) was thawed at room temperature

and added to tube containing dried DNA and mixed by pipetting. 200 µl of solution II (supplied with the kit) were added and mixed by vortexing. Cells were incubated at 30 ℃ for I hr and vortexed every 15 minutes. Cells we re subjected to heat shock at 42 ℃ for 15 minutes and 1 ml YPD medium was added. Af ter incubation at 30 ℃ for 1 hr (vortex every 15 minutes), cells were centrifuged at 1500 xg for 5 minutes. Cells were washed with 500 ml solution III (supplied with the kit), and centrifuged at 1500 xg for 5 minutes. The transformed cells were resuspended in 50 µl solution III. One aliquot of native cells (500 µl) of identical strain as competent cells was thawed, washed with 500 µl Solution III and centrifuged at 1500 xg for 5 minutes. Native cells were resuspended in 50 µl solution III and added to the transformed cells. After vortexing, the cell mixture was spread on YPD agar medium containing 100 µg Zeocin^{TM}. Plates were incubated 2-4 days at 30 ℃.

3.2.1.12 Colony PCR

Briefly, to perform colony PCR, primer seq2 (5´-GCAGCTCGCTCATTCCAATTCC-3´) was used as promoter specific primer, however, specific sense primers for P1, P2, P5 and HA1 were used as antisense primers (Table 1). Swabs from selected clones were suspended in dH_2O and heated 95 ℃ for 10 min, followed by centrifugation for 5 min at 4000 rpm. 10 µl of the supernatant was used to perform PCR. PCR reaction was done in a 20 µl final volume contains on 2 µl 10x polymerase buffer, 1 µl $MgCl_2$, 0.5 µl dNTP (10 mM), 0.5 µl of each primer (10 µM), 0.25 µl DNA Taq polymerase (2.5 U / µl), 10 µl DNA template (200 ng / µl) and 5.25 µl deionised water. The cycler program was 95 ℃, 2´´, 30x (95 ℃, 45´´; 56 ℃, 45´´ ; 72 ℃, 1´´), 72 ℃,10´´. PCR products were analysed by agarose g el electrophoresis.

3.2.1.13 Small- scale expression

Small scale expression was done to identify and confirm a recombinant *pichia* clones that express the correct protein and also to optimize the condition of expression. Primary culture was done from GS115 or SMD1168H *Pichia* cells in YP 2 % Gly using micro-titre plate 24 wells and incubated at 28 ℃ for 24 hrs at 250 rpm. An ensuing preparatory culture was initiated when OD_{600} = 0.8 - 1.0 and incubated at 28 ℃ for 24 hrs at 250 rpm. Induction of expression w as done in YP 2 % MeOH either at pH 6 or 8 with OD_{600} = 30 - 70. Protein expression was analysed 12, 24, 48 and 72 hrs after induction.

3.2.1.14 Mid-scale expression

After confirming the desired protein in supernatant, the expression conditions were optimized. For production of large amounts of protein, expression was done in mid - scale using shaking flasks.

3.2.1.15 Large scale expression

Recombinant HA1 polypeptide was expressed in *P. pastoris* using BIOSTAT Cplus-C15-3 fermenter to establish a high-density cell fermentation method. The culture temperature in pre-induced stage was optimised at 28 ℃ to adapt cell growth and recombinant protein expression in YP 2 % Gly at pH 6. Induction was done in the same media (at OD_{600} = 40) using 2 % methanol after adjusting pH to be 8.

3.2.1.16 Protein extraction from yeast cells

50 µl of yeast cells (nearly 10^9 cells) were resuspended in 1 ml cold distilled water followed by centrifugation 17900 xg for 1 min. Pellet was resuspended in 1 ml cold distilled water and 160 µl of a solution containing 1.85 M NaOH and 7.4 % mercapto-ethanol were added. The tube was inverted and incubated on ice for 10 min. 160 µl 50 % TCA were added and the tube was inverted and centrifuged 17900 xg for 5 min. The pellet was washed with 1 ml cold acetone, centrifuged 17900 xg for 5 min and dried in speed vacuum for 2 min. For Western blot or SDS-PAGE, 80- 100 µl 2X tricine sample buffer was added and boiled for 5 min at 95 ℃ before loading the sample onto polyacrylamide gel.

3.2.1.17 Purification of recombinant polypeptides

All purification steps were performed at room temperature. The purification protocol was optimised for each polypeptide using nickel-nitrilotriacetic acid (Ni-NTA) or lectin affinity chromatography (lectin peroxidase concanavalin). Briefly, for nickel affinity chromatography, column was prepared according to the manufacture protocol using 4 ml Ni-NTA agarose. The column was equilibrated by passing 10 ml equilibration buffer (wash buffer 1 or 2). Culture supernatant containing peptides of interest were passed through the column and washed with 10 ml washing buffer (wash buffer 1 or 2). Elution was done by increasing the concentration of imidazole (elution buffer 1) or decreasing the pH (elution buffer 2). For lectin affinity chromatography, equilibration was done by wash buffer 3. The unbound fraction was collected and the column was washed with 10 ml wash buffer 3. The bound fraction was eluted from the column with 0.4 M methyl α- D- manopyranoside in equilibration buffer (elution buffer 3). The fractions were dialyzed and concentrated by using vivaspin ultrafilter with a molecular

weight cut-off of 5,000 – 50.000 Da according to the size of polypeptide. Alternatively, purification was done under native or denaturing conditions using 4 M urea and 1 mM PMSF.

3.2.1.18 SDS- PAGE

P1, P2, P5 and rHA1 were separated by SDS-PAGE and stained by silver stain according to Nesterenko and co-workers (NESTERENKO et al. 1994). Proteins were separated by SDS-PAGE 10 % gels (Tables 2). Briefly, the SDS-PAGE apparatus was assembled then the separating gel was prepared and poured into the chamber up to the desired mark; the rest of the chamber was filled by adding distilled water. After polymerisation, the gel was dried by soaking with filter paper. The collecting gel was prepared and poured up on the separating gel. The selected comb was introduced to make the required numbers of slots. The gel is allowed to stand at room temperature until polymerisation. The gel was transferred into the electrophoresis chamber and running buffers (anode and cathode buffers) were added. Protein samples were mixed at a 1:1 ratio with 2x tricine sample buffer and heated 95 ℃ for 5 minutes. 10-30 µl of the test sample along with the marker were loaded in respective slots with a special 100 µl syringe and a needle. Then the electrophoresis container was connected with a power source at 160- 180 V for 45 min -1 hr. After electrophoresis, the gel was either subjected to silver stain (Table 3) or Western blot.

Table (2): Composition of polyacrylamide gel

Solution	**Separating gel (10 %)**	**Stacking gel (4 %)**
Acrylamide (30 %)	1.63 ml	0.27 ml
Tris / Cl / SDS pH 8.45	1.67 ml	0.52 ml
Dist. H_2O	1.17 ml	1.3 ml
Glycerine	0.53 ml	-
10 % APS	16.67 µl	8.3 µl
TEMED	3.4 µl	3.4 µl

Table (3): Silver staining of recombinant polypeptides

Step	Treatment	Time
Fixation	60 ml acetone (50 %), 1.5 ml TCA (50 % w/v) and 25 µl formaldehyde 37%	5 min
Rinsing	dH_2O	3X 5s
Washing	dH_2O	5 min
Rinsing	dH_2O	3X 5s
Pre-treatment	60 ml acetone 50 % (v/v)	5 min
Pre-treatment	100 µl sodium thiosulfate (10 % w/v) in 60 ml dH_2O	1 min
	dH_2O	3 X 5s
Impregnation	0.8 ml silver nitrate (2% w/v), 0.6 ml formaldhye 37 % in 60 ml dH_2O	8 min
Rinsing	dH_2O	2X 5s
Development	1.2 gm sodium carbonate, 25 µl formaldhyde and 25 µl sodium thiosulfate (10 %) in 60 ml H_2O	10- 20s
Stopping	60 ml acidic acid	30s
Rinsing	dH_2O	10s

3.2.1.19 Western blot

For western blot, proteins were transferred on nitrocellulose membrane (Roti®-PVDF) according to Towbin and co-workers (TOWBIN et al. 1979)**.** The blotting was performed in transfer buffer at 160 mA for 1 hr followed by 3 times washing with 1X PBS, pH 7.4. The membrane was blocked by incubation in 1X PBS containing 4 % skim milk powder for 15 min at room temperature. For analysis of His-tag, the blocked membrane was incubated overnight with 1:200 mouse anti-His-tag. After washing with 1X PBS, the membrane was incubated with secondary antibody in a concentration of 1: 1000 (polyclonal rabbit anti-mouse IgG HRP conjugate) at room temperature for 2 hrs. The membrane was washed with 1X PBS and developed with 1X PBS containing 5 mg of DAB and 10 µl H_2O_2 30 %.

3.2.1.20 MALDI-TOF

The ZipTip pipette tip is a 10 µl pipette tip with a bed of a chromatography media fixed at its end. It is intended for concentration and purifying peptide to be analyzed by MALDI-TOF. Sample preparation was done according to manufacturer, briefly; the pH of protein sample (10-20 µl) was adjusted to be less than 4 using 0.1 % TFA in

Milli Q®. The ZipTip pipette tip was equilibrated twice in 100 % ACN followed by twice in 0.1 % TFA. Protein was aspirated and dispensed 7-10 cycles. The ZipTip pipette tip was washed twice by aspirate and dispensing 0.1 % TFA. Elution was done by using varying concentrations of ACN (20 %, 30 %, 50 % and 70 %). The sample was dried by vacuum centrifuge and analyzed by MALDI-TOF in Institute of Biochemistry, Faculty of Medicine, Leipzig University.

3.2.1.21 Glycosylation analysis

3.2.1.21.1 Blot with concanavalin

Glycosylated polypeptides (P2, P3 and rHA1) were separated by SDS – PAGE and transferred to nitrocellulose. The membrane was blocked by incubation in 1X PBS containing 2 % (v/v) Tween20 for 2 minutes. The blot was rinsed twice in 1X PBS followed by incubation with 10 µg / ml of lectin peroxidase (lectin from concanavalin) in PBS containing 0,05 % (v/v) Tween20, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ for 16 hrs at 20 ℃. The blot was rinsed in 1X PB S and developed using DAB as described in Western blot.

3.2.1.21.2 Deglycosylation with endoglycosidase

Deglycosylation was done using Endoglycosidase H kit (Endo H_f). Briefly, 1 µl of 10X glycoprotein denaturating buffer (supplied with the kit) was added to 9 µl of purified HA1 then denatured at 100 ℃ / 10 min. 4 µl 10X GS reaction buffer (supplied with the kit) was added. Deglycosylation was done by incubation of the mixture with Endo H_f at a concentration of 1: 500. Deglycosylation was analysed after 0 min, 15 min, 30 min, 1 hr, 2 hrs, 3 hrs and 4 hrs by SDS-PAGE, Western blot and blotting using concanavalin.

3.2.1.21.3 Expression of rHA1 in the presence of tunicamycin

rHA1 was expressed in YP 2 % MeOH (v/v) in the presence of tunicamycin (1 to 100 mg / ml). In vitro characterization of protein expression was done by SDS-PAGE followed by Western blot.

3.2.1.22 Protein precipitation

3.2.1.22.1 TCA protein precipitation

To concentrate proteins for analysis by Western blot, 10 % (v/v) of TCA 100 % was added to yeast culture supernatant and incubated on ice 1 hr at - 20 ℃ followed by centrifugation at 17900 xg / 5 min. The pellet was washed with cold acetone 100 % and centrifuged at 17900 xg / 5 min. The sample was dried under vacuum to

eliminate any acetone residue. For Western blot, 2X tricine sample buffer was added, sample and boiled 95 ℃ for 5 min before loading on to polyacrylamide gel.

3.2.1.22.2 Acetone precipitation

Four parts of 100 % acetone were added to protein sample and incubated 4 ℃ for 1 hr followed by centrifugation at 4000 rpm / 45 min / 4 ℃. The sample was dried under vacuum (speed-vac) to eliminate any acetone residue. For Western blot, after addition of 2X tricine sample buffer, the sample was boiled 95 ℃ for 5 min before loading onto polyacrylamide gel.

3.2.1.22.3 Ultracentrifugation

Yeast culture supernatant was concentrated using vivaspin ultrafilter. Different sizes of ultrafilters were used (5,000 – 60,000 Da) according to the protein size.

3.2.1.22.4 Ammonium sulfate precipitation

For purification, protein was precipitated using ammonium sulfate. Slowly, solid ammonium sulfate was added to a final concentration of 80 % (470 g / litre of solution) and stirred at 4 ℃ for 15 min. The sample was centrifuged by ultracentrifugation (Surespin Rotor, 11000 xg / 30 min / 4 ℃). The pellet was resuspended in an appropriate volume of equilibration buffer that used in purification.

3.2.1.23 Determination of protein concentration

Protein quantitation was determined by colorimetric method, using Bradford™ assay kit. BSA with known concentration was used as a standard. Briefly, 25 µl of standard BSA or unknown sample was pipetted to 25 µl millipore H_2O and serial dilution was done. 10 µl of each dilution was added to a 96 - micro-titre plate containing 200 µl of diluted dye with millipore water at a ratio of 1:4. Plates were incubated for 30 minutes at room temperature in dark place. The absorbance was measured at OD_{590} nm. The protein concentration was calculated by comparing with the protein standard curve.

3.2.1.24 Cell passage

Vero cells were grown in 25 or 75 cm^2 plastic flasks in DMEM + GlutaMax™, supplemented with 5 % FCS at 37 ℃ in a 5 % CO_2 humidified incubator. When monolayer was confluent in the flask, the media was removed and the cells were washed with sterile 1X PBS (PBS 10x - Ca^{2+}, - Mg^{2+}), then 3 ml of 0.05 % trypsin-EDTA were added to the 25 cm^2 flask and incubated 2 - 5 minutes at 37 ℃ to dislodge the cells from the flask. Trypsin was inhibited by addition of 7 ml DMEM + GlutaMax™ containing 5 % FCS. The cells were passaged as before using a split ratio of 1: 10.

3.2.1.25 Virus propagation

Influenza A subtype H5N1 (A / Thailand / 1 (Kan-1) / 2004) was propagated in Vero cells. When the Vero cells formed a monolayer, the medium was discarded and the cells were washed with sterile 1X PBS (3 ml / 25 cm^2 flask and 6 ml / 75 cm^2 flask). Aliquots of 50 - 150 µl of diluted influenza virus in 2-5 ml DMEM medium (DMEM + GlutaMax™) were inoculated onto monolayer cells. Cells were incubated for 60 - 90 minutes at 37 ℃ in a 5 % CO_2 humidified incubator, followed by addition of 8 - 25 ml DMEM medium containing 5 % FCS. The cells were incubated at 37 ℃ in a 5 % CO_2 humidified incubator for 5 days and observed daily for cytopathic effect (CPE). Virus growth was confirmed by virus haemagglutination.

3.2.1.26 Virus titration

Serial 10 fold dilutions of influenza A subtype H5N1 (A / Thailand /1 (Kan-1) / 2004) stock solution were made in DMEM + GlutaMax™ medium. Titration was done in 96-well cell culture plate containing Vero cells monolayer. The medium was discarded and cells were washed with sterile 1X PBS (PBS without Ca^{2+} and Mg^{2+}). 100 µl diluted virus was added to each well and incubated for 1 hr at 37 ℃ in a 5 % CO_2 humidified incubator. 100 µl of DMEM + GlutaMax™ containing 5 % FCS was added and incubated 37 ℃ in a 5 % CO_2 humidified incubator for 5 days. The medium was removed and cells were stained by 0.1 % crystal violet. The titre was expressed as 50 % tissue culture infective dose / 0.1 ml ($TCID_{50}$) and was calculated by the method of Reed-Muench (REED and MUENCH 1938)

3.2.1.27 Virus inactivation and purification

H5N1-virus was propagated on Vero cells. After 5 days, supernatant was harvested, clarified (340 xg / 10 min.) and inactivated using ultraviolet irradiation (30 W / G30T8) for 1hr. The clarified supernatant was layered in 25 % sucrose cushion in TNE buffer and centrifuged by ultracentrifugation (Surespin Rotor, 11000 xg / 4 ℃ / 4 hrs). The pellet was resuspended in TNE buffer and the virus (100 $TCID_{50}$ / 100 µl) was tested for its safety in Vero cells. Fractions were pooled and the virus was titrated by haemagglutination test using 1 % chicken red blood cells (RBCs). This preparation was used for chicken vaccination and as antigen in serological tests.

3.2.2 Immunogenicty of recombinant polypeptides in mice and chickens

3.2.2.1 Immunogenicity of recombinant polypeptides in mice

The immunogenicity of P1, P2 and rHA1 was evaluated in BALB/c Mice. Three groups of 2 mice were immunized subcutaneously with 50 µl Gerbu adjuvant. Two immunization protocols were used as shown in the following table. Another group of 2 mice was injected with adjuvant in 1xPBS. Sera were obtained from euthanized mice one week after the 2nd and at the end of experiment from orbital sinus. Evaluation of the immune response was done by recombinant Elisa (using recombinant polypeptides), whole H5N1 Elisa and IFA.

Immunization	Immunization protocol (IP1)	Immunization protocol (IP2)
Priming	0	0
1st booster	14	21
2nd booster	21	28
3rd booster	28	35
4th booster	35	42
5th booster	42	49
Final Bleeding	45	52

3.2.2.2 Immunogenicity of recombinant polypeptides in chickens

The immunogenicity of P1, P2, P5 and rHA1 polypeptides was evaluated also in inbred commercial layer chickens in comparison with prepared inactivated H5N1 (A / Thailand / 1 (Kan-1) / 2004) antigen. Groups of 4 chickens were injected intramuscularly with 100 µg of recombinant polypeptide mixed with 100 µl Gerbu adjuvant. Two weeks post priming; chickens were boosted 3 times, at weekly intervals using the same amount of antigen and adjuvant that used for the initial immunization. Chickens immunized with inactivated vaccine were injected with a dose of 8 HA unit (HAU) mixed with 100 µl Gerbu adjuvant. Control chickens were injected with adjuvant only. Sera were obtained at day 0 as well as 4, 5, 6 and 8 weeks post primary vaccination from wing vien and analyzed for specific antibodies with recombinant Elisa (using the same antigen used in vaccination), whole H5N1 Elisa (using whole H5N1 antigen), AGID, IFA and µNT. Moreover, IgY was analysed in egg yolk collected at day 0 as well as 4, 5, 6 and 8 weeks post primary vaccination by recombinant Elisa, whole H5N1 Elisa and IFA.

Table (4): Experimental design for assessment of immunogenicity of recombinant polypeptides in chickens compared with inactivated H5N1

Group No	Vaccination regime			Assessment of immune response
	Vaccine type	Vaccine dose	Frequency	
1	rHA1	100 µg	4X	rElisa[a]
2	P1	100 µg	4X	whole H5 Elisa[b]
3	P2	100 µg	4X	AGID[c]
4	P5	100 µg	4X	Microneutralization[d]
5	Inactivated H5	8 HAU	4X	IFA[e]
6	Adjuvant vaccinated	-	4X	

[a]Elisa plates were coated with homologous antigen used in vaccination (50 ng / well).
[b]Elisa plated were coated with inactivated H5N1 (A / Thailand / 1 (Kan-1) / 2004) antigen (1 HAU / well).
[c]AGID was done according to (BEARD 1998) using H5N1 (A / Thailand / 1 (Kan-1) / 2004) antigen.
[d]Microneutralization test was done according to (ROWE et al. 1999) using H5N1 (A / Thailand / 1 (Kan-1) / 2004) virus.
[e]Immunofluorescence assay was done on Vero cells infected with H5N1 (A / Thailand / 1 (Kan-1) / 2004) virus.

3.2.2.3 Extraction of IgY from eggs

Extraction and purification of IgY was done according to (POLSON et al. 1980). Briefly, individual yolk was separated from the egg white and washed with PBS. 400 µl of egg yolk was obtained and mixed with 800 µl PBS. Polyethylene glycol (PEG), molecular weight 1500, was added to a final concentration of 3 % (w/v). After incubation for 20 min at room temperature the mixture was centrifuged at 14,000 xg for 10 min. The fatty layer was removed and the water soluble supernatants with the pellet were collected in another tube. PEG was added to a final concentration of 12 % and the mixture was centrifuged at 14,000 xg for 10 min. The pellet was

resuspended in ethanol 50 % (v/v in dist. H_2O). After centrifugation at 10,000 xg / 30 min / 4 ℃, the pellet was resuspended in 200 µl dist. water. Extracted IgY was analysed by SDS-PAGE. H5 specific antibodies were analyzed by recombinant Elisa, whole H5N1 Elisa and IFA.

3.2.2.4 Elisa

Recombinant peptide (50 ng / well) or inactivated whole H5N1 (1 HAU / well) was coated in duplicate onto 96–well microtiter plate (microlon high binding). The working dilution of HRP-conjugate rabbit anti-mouse IgG and HRP rabbit anti-chicken IgG in PBS, pH 7.4 supplemented with 0.05 % (v/v) Tween20, 3 % (w/v) BSA) were 1:2,000 and 1: 20,000, respectively. For Elisa, plates were coated with 50 µl antigen solution diluted in coating buffer (200 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4 ℃. Plates were washed (30 seconds) five times with washing buffer (PBS, 0.05 % Tween20) and blocked for 1 hrs at 37 °C with 300 µl / well blocking buffer (PBS, 0.05 % Tween20, 3 % BSA). Sera were diluted in blocking buffer and 50 µl of the dilution was added to each well in duplicate. After 2 hrs incubation at 37 ℃, and 5X washing, 100 µl HRP-labelled (anti-mouse or anti-chicken IgG) antibody was added and incubated 37 ℃ for 90 minutes. Plates were then washed 5X and colour development was accomplished by adding 100 µl / well of 3,3´,5,5´tetramethylbenzidin (TMB) in sodium acetate-citrate buffer with 2 µl 30 % hydrogen peroxide (H_2O_2). After 20 minutes incubation in the dark at room temperature, the reaction was stopped by addition of 50 µl Sulfuric acid (H_2SO_4, 0.5 M). The optical density (OD) was read at 450. Antibody titres were expressed as the serum dilution at which half-maximal OD_{450} readings of serum (half-maximal OD_{450}).

3.2.2.5 Agar gel immunodiffusion test

The test was performed as described by Beard (BEARD 1998)**.** The test was carried out using 1 % (w/v) agarose and 8 % (w/v) NaCl in 1X PBS, PH, 7.2. The medium was poured to a thickness of 2- 3 ml in petri dishes. Six peripheral wells surrounding a central well in a hexagonal form were made in the agar medium, the well size was 4 mm in diameter, and the distance between the central well and the evenly spaced peripheral wells was 4 mm. Thailand H5N1 virus was used as antigen. Sera to be tested for precipitating antibody were placed into the peripheral wells while the antigen was placed into the central well. Final readings were recorded as negative (-) or positive (+) after 48 hrs.

3.2.2.6 Micro neutralization test (µNT)

µNT was carried out according to Rowe and coworkers (ROWE et al. 1999). Briefly, serum samples were diluted with cell culture medium in two fold steps. The dilutions were mixed at a ratio of 1:1 with H5N1 influenza virus (10 $TCID_{50}$ per well), incubated for 1 hr at room temperature and transferred to a microtiter plate with a Vero cell monolayer. Plates were incubated 18 hrs at 37 ℃. A fter removal of medium, plates were washed with PBS and fixed with 80 % acetone in sterile dist. H_2O for 30 min. After blocking with PBS containing 1 % BSA and 0.1 % Tween20, 50 µl of influenza anti-nucleoprotein was added and incubated 1 hr at 37 ℃. IgG HRP rabbit anti-mouse antibody (1: 2,000) was added and plates were incubated 1 hr at 37 ℃. Freshly prepared substrate was added and incubated 30 minutes at room temperature in the dark. Stop solution (H_2SO_4, 0.5 M) was added and the absorbance OD of the wells was read at 450 nm. The endpoint titre was expressed as the reciprocal of the highest dilution of serum with OD_{450} value above the mean + (3 x standard deviation) of the negative control.

3.2.2.7 Immunofluorescence assay (IFA)

For detection of influenza-H5 specific antibodies, Vero cells were propagated on cover slips in a 6-well plate and infected at 0.01 multiplicity of infection (m.o.i.) with A / Thailand / 1(Kan-1) / 2004 isolate. After 24 hrs incubation at 37 ℃, in a 5 % CO_2 humidified incubator, cells were fixed for 30 minutes with ice cold 80 % acetone in sterile distilled water at -20 ℃. Fixed cells were blocked for 30 minutes with 5 % PBSA (PBS, pH 7.4 contains 5 % BSA) followed by incubation for 90 minutes with diluted chicken sera or mouse sera at 37 ℃. After washing with 0.5 % PBSA, cells were incubated for 45 minutes with FITC labelled monoclonal anti-chicken IgY or anti-mouse IgG (at a dilution of 1: 100). After 5 times washing with 0.5 % PBSA, cells were rinsed again in dH_2O. The cover slip was inverted, mounted onto a glass slide using mounting fluid (1x PBS, 10 % glycerine) and observed for fluorescent staining under fluorescence microscopy.

3.2.3. Development or recombinant Elisa for detection of influenza subtype A H5 antibodies

3.2.3.1 Experimental design

For this purpose 179 serum samples were obtained from commercial broiler chickens (see materials 3.1.7). The work design is shown in the following diagram.

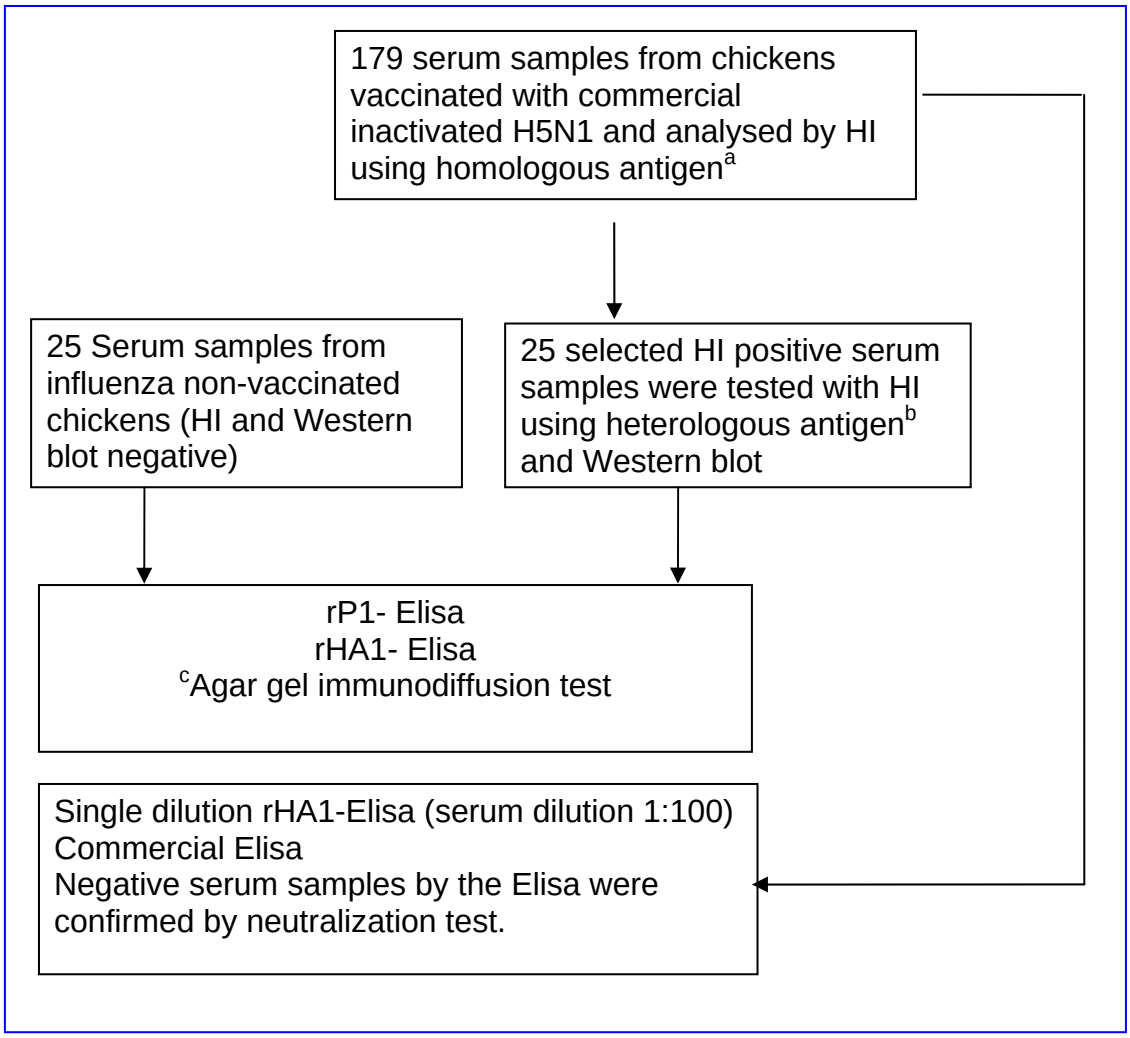


Figure (3): Flow chart to study the validity of recombinant Elisa for detection of H5 antibodies in chicken sera.

[a]HI was done using homologous commercial H5N2 (A / chicken / Mexico / 232 / 94 / CPA) antigen.

[b]HI was done using heterologous H5N1 (A / Thailand / 1 (Kan-1) / 2004) antigen

[c]AGID test was done using heterologous H5N1 (A / Thailand / 1 (Kan-1) / 2004) antigen.

3.2.3.2 Haemagglutination inhibition test

Chicken sera were examined for haemagglutination inhibiting antibodies by HI test, according to OIE manual (OIE 2005), using commercial H5N2 antigen (A / chicken / Mexico / 232 / 94 / CPA) or H5N1 (A / Thailand / 1 (Kan-1) / 2004) antigen.

Haemagglutination test

HA test was used for the titration of commercial and H5N1 Thailand isolate antigen before the HI test. Lyophilized H5N2 (A / chicken / Mexico / 232 / 94 / CPA) antigen was dissolved by 1 ml sterile PBS (pH, 7.2). For titration of influenza antigens, 25 µl of PBS were placed into each well of a plastic V-bottomed microtitre plate. 25 µl of virus suspension were placed in the first well and a two-fold dilution of virus suspension was done. A further 25 µl of PBS were dispensed to each well then 25 µl of 1 % (v/v) chicken RBCs were added to each well and the RBCs was left to settle at room temperature by which time control RBCs should be settled to a distinct button. The titration was read to the highest dilution giving complete HA, representing 1 HA unit (HAU).

Haemagglutination inhibition test

25 µl of PBS was dispensed into each well of a plastic V-bottomed microtitre plate. 25 µl of serum was added to the first well of the plate and a two-fold dilution of the serum was done across the plate. 4 HAU of tirtrated influenza antigen in 25 µl was added to each well and plates were incubated for a minimum of 30 minutes at room temperature. 25 µl of 1 % (v/v) chicken RBCs was added to each well and after gentle mixing, RBCs was left to settle, by which time control RBCs should be settled to a distinct button. The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The HI titres were determined as reciprocals of highest serum dilutions in which inhibition of haemagglutination was observed.

3.2.3.3 Western blot for analysis of antibodies in serum

For analysis of individual serum by Western blot, HA1 polypeptide was separated by SDS-PAGE and transferred on nitrocellulose membrane as mentioned before. After blocking, the membrane was cut into strips and incubated with 1:100 dilution of serum at 4 ℃ overnight. After washing with 1xPBS, the membrane was incubated with IgY HRP rabbit anti-chicken conjugate in a concentration of 1: 10000 at room temperature for 2 hrs. The membrane was washed with 1xPBS and developed as mentioned before.

3.2.3.4 Recombinant Elisa

Recombinant Elisa was done as described in 3.2.2.4 using 2 recombinant polypeptides (P1 and rHA1).

3.2.3.5 Commercial Elisa

The test was employed for detection of AI antibodies in chicken sera using commercial ELISA kits as described by manufacturer. For individual bird interpretation, titres ranging from 0 to 269 considered negative and ≥ 300 were considered positive. Valid AI-ELISA were obtained when the average optical density value of the normal control serum is less than 0.200 and corrected positive control value range is between 0.250 and 0.900. Samples testing with a sample / positive value (SP) value of less than or equal to 0,150 received a 0 titre value. Under optimal conditions (room temperature 21-24 C), the optical density values ranges from 0.060 to 0.080 for AI normal control serum and 0.400 to 0.750 for AI positive control serum.

3.2.3.6 Neutralization test

Serum samples which are negative by both rHA1 and/or cElisa were analyzed by NT according to Rowe and co-worker (ROWE et al. 1999). After 3 days incubation at 37 ℃, plates were inspected as positive or negative a ccording to the presence or absence of cytopathic effect (CPE). Positive control sera were included as positive control.

3.2.3.7 Validity of rHA1 Elisa for detection of H5 antibodies in duck serum

Duck sera were tested using rHA1 in comparison with HI. rHA1-Elisa was performed as described before; however, goat anti-bird IgG HRP was used as a secondary antibody.

3.2.3.8 Statistical analysis

Relative sensitivity and specificity of rElisa in comparison with other performed serological tests were calculated according to Mohan and co-workers (MOHAN et al. 2006).

Calculation of relative sensitivity and specificity

Test A	Test B		Total
	Positive	Negative	
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	a+b+c+d

Relative sensitivity = a / a + c

Relative specificity = d / b + d

Determination of agreement ratio

The formula of JIN et al. (2004) was employed to compare differences among the Elisa (either commercial or recombinant) and HI test in evaluating sera obtained from experimentally vaccinated commercial broiler. The formula is given by the function:

$$\text{Agreement ratio} = \frac{A + B \; x \; 100 \; \%}{C + D}$$

Where: A = the total number of positive sera examined by HI test subtracted from the total number of positive sera examined by Elisa test.

B = the total number of negative sera examined by HI test subtracted from the total number of negative sera examined by Elisa test.

C = total positive number of sera examined by Elisa test.

D = total negative number of sera examined by Elisa test

4. RESULTS

4.1 Expression of recombinant polypeptides in *P. pastoris*

4.1.1 PCR

The coding sequences of genes of interest were isolated and amplified from influenza A subtype H5N1 (A / Thailand / 1(Kan-1) / 2004) using primers designed with Xho1 and Not1 restriction sites (Figure 4). The size of the H5-P1, H5-P2, H5-P5 and H5-HA1 coding sequences were 159, 329, 128 and 1007 bp, respectively.

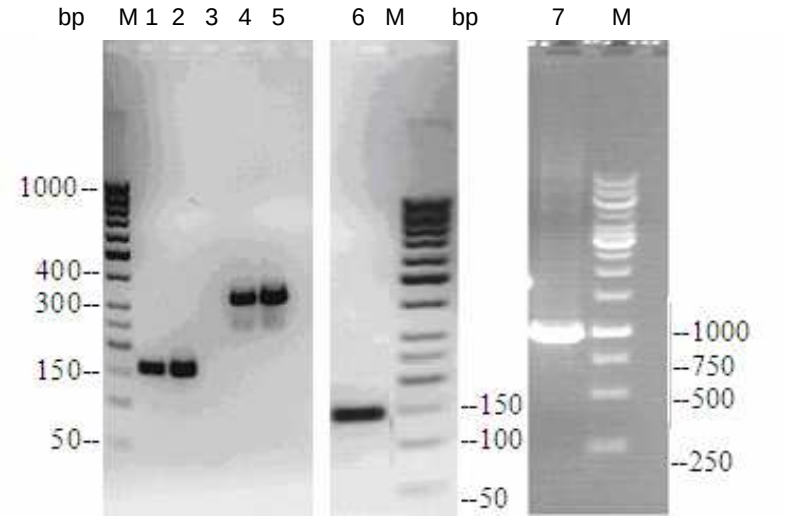


Figure (4): PCR amplification of truncated sequences of influenza A subtype H5N1 (A / Thailand / 1(Kan-1) / 2004). Lanes (1 and 2): amplification of P1, lane (3): negative control. Lanes (4 and 5): amplification of P2. Lane (6): amplification of P5. Lane (7): amplification of HA1.

4.1.2 Molecular cloning

PCR products were digested using Xho1 and Not1, purified and ligated with pAOX. After transformation of pAOXH5-P1, pAOXH5-P2, pAOXH5-P5 and pAOXH5-HA1 to *E-coli* cells, many colonies arose on selecting plates. Single colonies were cultured in liquid medium (LS-LB) for plasmid isolation. Double digestion of pAOXH5-P1, pAOXH5-P2, pAOXH5-P5 and pAOXH5-HA1 resulted in large segments (2614, 2400, 2454 and 2799 bp, respectively) and small segments (1224, 1206, 1183 and 1716 bp, respectively) (Figure 5). Plasmids containing genes of interest were sequenced to confirm the presence of desired sequences. Linearized plasmids were analyzed on agarose gel electrophoresis (Figure 6).

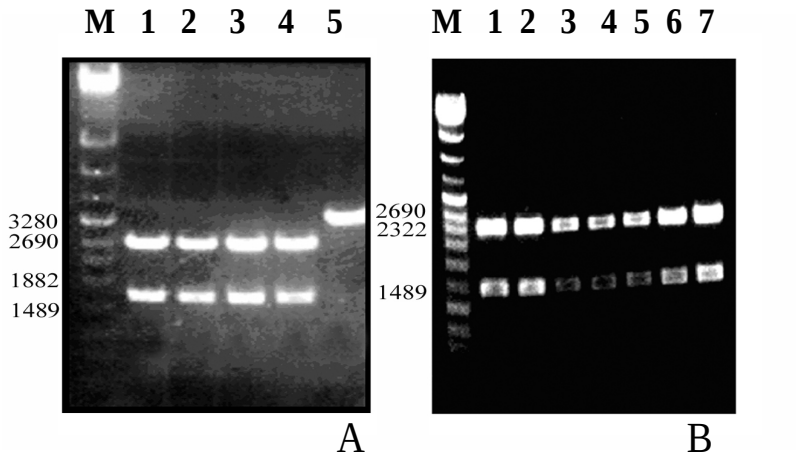


Figure (5): Analysis of plasmids containing gene of interest.

A) Double digest of pAOX-HA1 using Bgl II restriction enzyme resulting in a large segment (2799 bp) and a small segment (1716 bp). Lanes 1-4 different clones subjected to double digest. Lane 5: intact pAOX-HA1 plasmid. B) Double digest of pAOX-P5 using Bgl II and Xho1 restriction enzymes resulting in a large segment (2453 bp) and a small segment (1183 bp). Lanes 1-7 are different clones.

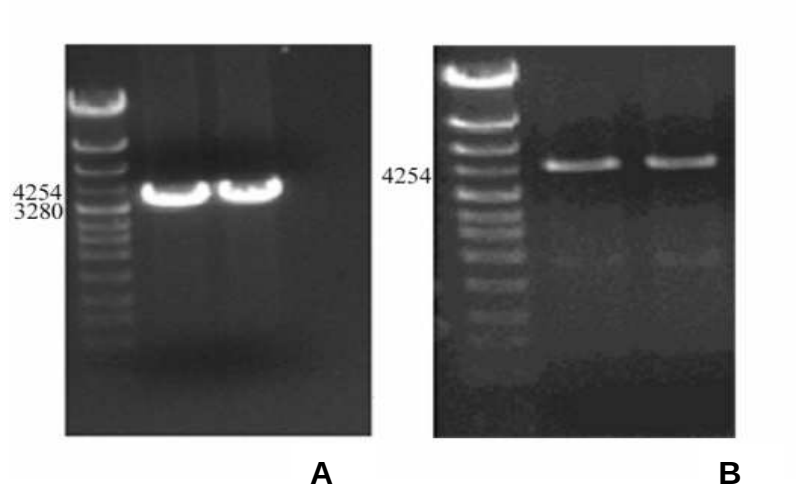


Figure (6): Plasmids linearization. A) Linearized pAOX-P5 after restriction digest with BstX1 (size 3636 bp). B) Linearized pAOX-HA1 after restriction digest with BstX1 (size 4515 bp).

4.1.3 *Pichia pastoris* transformation

Linearized plasmids were transformed successfully into *P. pastoris* either SMD1168H or GS115 strains. The colonies appear circular with a size of 1- 5 mm. The number of colonies ranged from 80 - 108 colonies in GS115 yeast cells and in case of SMD1168, the colonies number ranged from 10 – 20 colonies. Presence of the respective gene of interest was verified by colony PCR using gene specific primers and promoter specific primers (Figure 7).

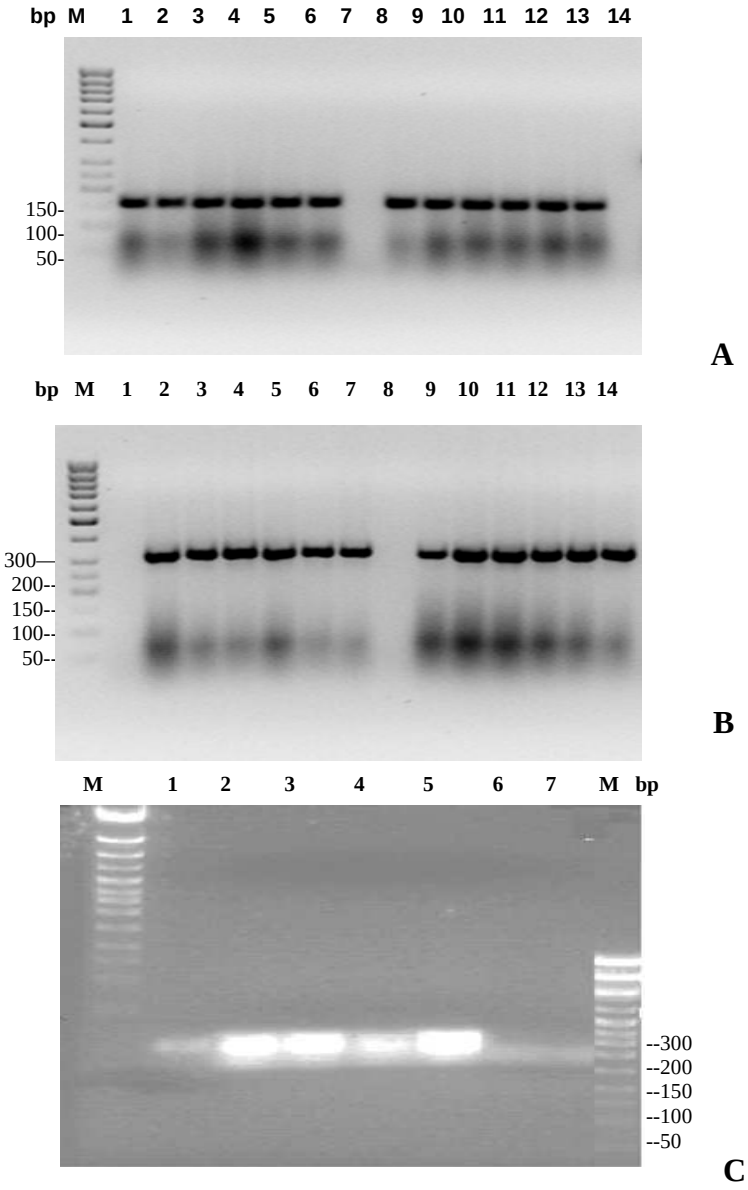


Figure (7): Verifying the gene of interest in positive selected transformants by colony PCR using gene specific and promotor specific primers.

A: Colony PCR of different P1 clones grown on SMD1168H (lanes 1-6) and GS115 (lanes 8-13). Lanes (7 and 14) are untransformed SMD1168H and GS115, respectively. Positive P1 transformants result in a 160 bp fragment.

B): Colony PCR of different P2 clones grown on SMD1168H (lanes 2-7) and GS115 (lanes 9-14). Lanes 1 and 8 untransformed SMD1168H and GS115, respectively. Positive P2 transformants result in a 330 bp fragment.

C): Colony PCR of different P5 clones grown on SMD1168H (lanes 1-5). Lanes (6 and 7) untransformed SMD1168. Positive P2 transformants result in a 251 bp fragment.

4.1.4 Expression and analysis of recombinant polypeptides

P1 and P2 polypeptides were expressed in YP 2 % methanol, pH 8 for 24 hrs / 29 ℃ / 250 rpm. Purification was carried out using Ni-NTA chromatography under natural condition (Figure 8.A). For P1 polypeptide, washing and elution were achieved by increasing imidazole to 100 mM (wash buffer 1 and elution buffer 1). Washing of P2 polypeptide was done by lowering pH to 6.5 and eluted at pH of 2-3 (Wash buffer 2 and elution buffer 2). P5 polypeptide was demonstrated in cell lysate of both GS115 and SMD1168H *Pichia* cells by SDS-PAGE and Western blot after induction in YP 2 % methanol, pH 6. The expected band was detected in supernatant of SMD1168H after expression in YP 2 %, pH 8. For purification, P5 polypeptide could not bind to Ni-NTA, neither under natural nor under denaturing condition. It was purified by lectin affinity chromatography using wash buffer 3 and elution buffer 3 (Figure 8.A). P1, P2 and P5 polypeptides were analyzed in culture supernatant 24 hrs after induction by Western blot using anti-His-tag antibodies (Figure 8.B). rHA1 polypeptide was demonstrated in cell lysate and supernatant of SMD1168H cells by SDS-PAGE and Western blot 24, 36 and 48 hrs after induction of expression, some protein degradation was observed as demonstrated by Western blot. Addition of 2 µg Pepstatin / ml prevented protein degradation. For purification, rHA1 polypeptide could not bind to Ni-NTA under natural condition; however, it binds under denaturing condition using 4 M urea. 1 mM PMSF was added to prevent the degradation during purification of rHA1 polypeptide. Washing was done using 10 mM imidazole (wash buffer 1). However, the desired rHA1 polypeptide was eluted using 100 mM imidazole (elution buffer 1). Analysis of rHA1 polypeptide by SDS-PAGE and Western blot showed a broad smear above the expected size (above 39.6 kDa). Analysis of rHA1 polypeptide treated with Endo H_f by SDS-PAGE and Western blot showed a band which is in accordance with the expected size (Figures 8.C). It reacted with lectin from Con A which reveals the presence of glycosylation (Figure 8.D). Expression of rHA1 in the presence of tunicamycin lead to partial deglycosylation but reduced the expression level. On the other hand, other glycosylated polypeptides (P2 and P5) could not react with Con A and could not deglycosylated with Endo H_f. MALDI-TOF analysis of P1 polypeptide showed a molecular weight of 7592.1 Da $[M+ H]^+$ compared to the theoretical mass: 7591,52 Da (Figure 9)

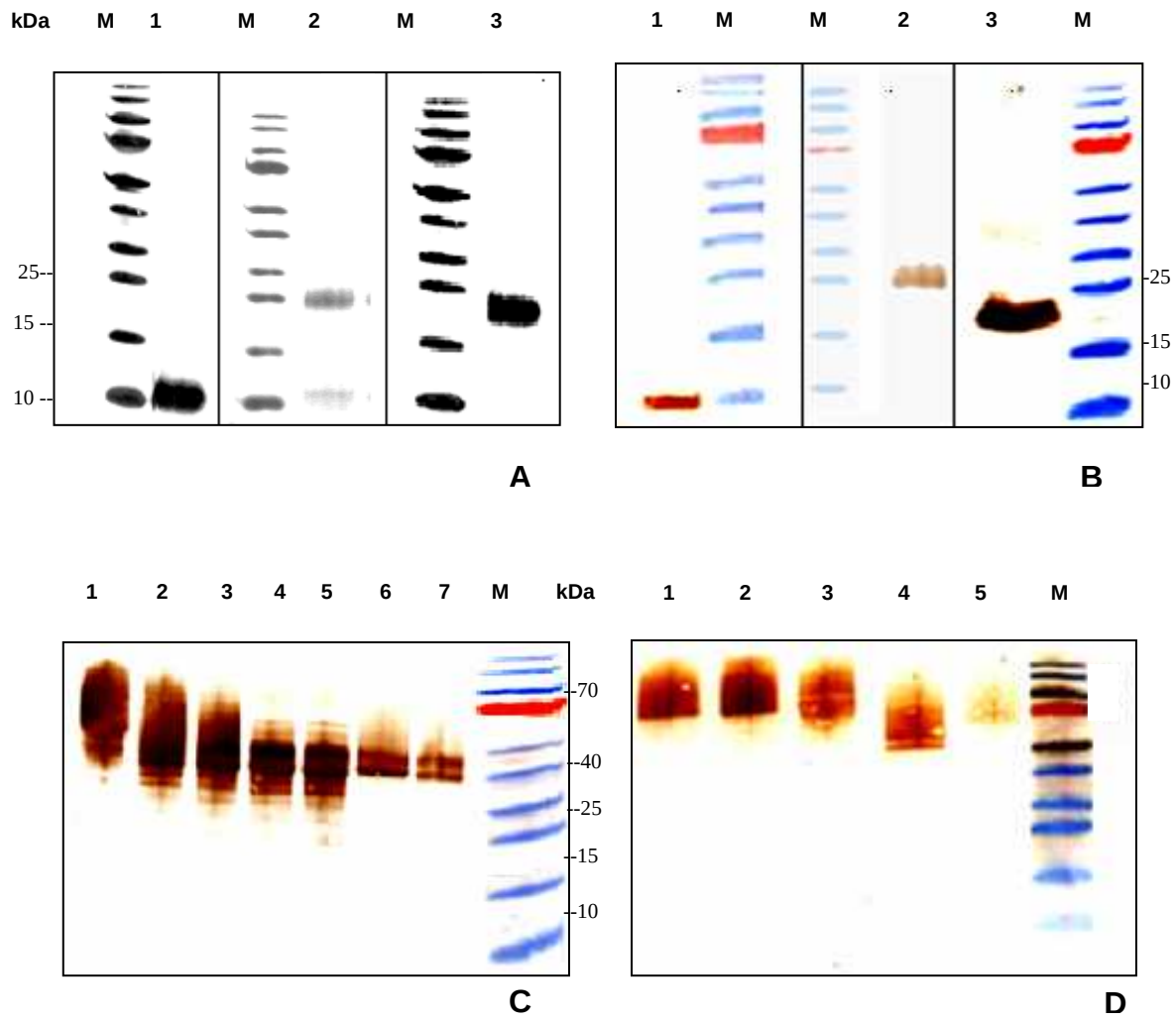


Figure (8): Analysis of recombinant polypeptides by SDS-PAGE and Western blot.

A and B): SDS-PAGE and Western blot analysis of purified P1, P2 and P5 polypeptides expressed in *P. pastoris* cells (strain SMD1168H) and secreted into supernatant, respectively. Lane 1): P1 polypeptide purified by nickel affinity chromatography, theoretical molecular mass 7.5 kDa. Lane 2): P2 polypeptide purified by nickel affinity chromatography, theoretical molecular mass is 13.9 kDa. Multiple bands are due to different glycosylation as P2 contains a glycosylation site. Lane 3) P5 polypeptide purified by lectin affinity chromatography, theoretical molecular mass is 6.5 kDa. The increase in size is attributed to presence of a glycosylation site.

C): Western blot analysis of rHA1 polypeptide. Lane 1: purified rHA1, Lanes 2, 3, 4, 5, 6 and 7 are purified rHA1 and treated with Endo H_f for 15 min, 30 min, 1hr, 2 hrs 3 hrs and 4 hrs, respectively.

D): Blot of rHA1 polypeptide using concanavalin. Lanes (1, 2 and 3): purified rHA1 polypeptide. Lanes 4 and 5: rHA1 treated with Endo H_f for 15 min and 2 hrs, respectively, and blotted using concanavalin.

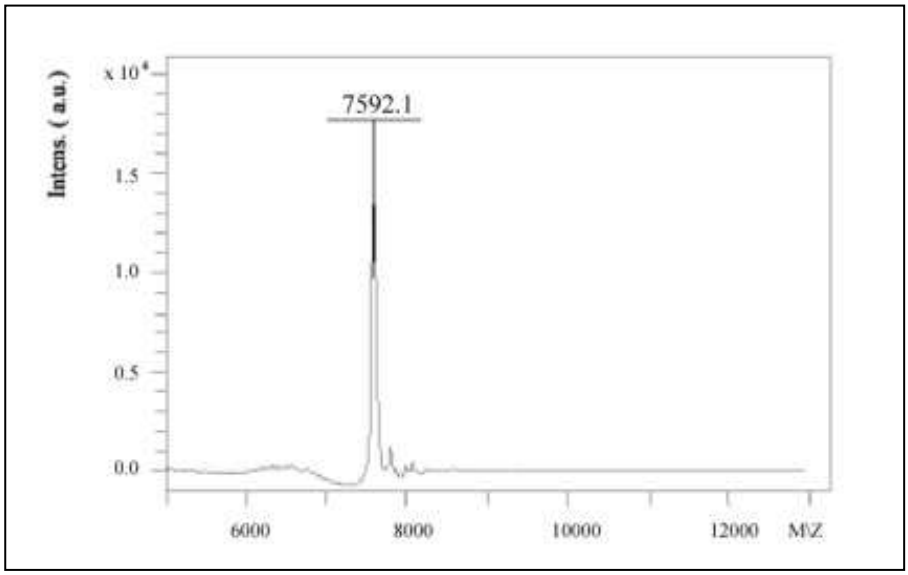


Figure (9): MALDI-TOF analysis of P1 polypeptide. The molecular mass of P1 polypeptide is 7592.1 Da [M + H]+ (theoretical mass: 7591,52 Da).

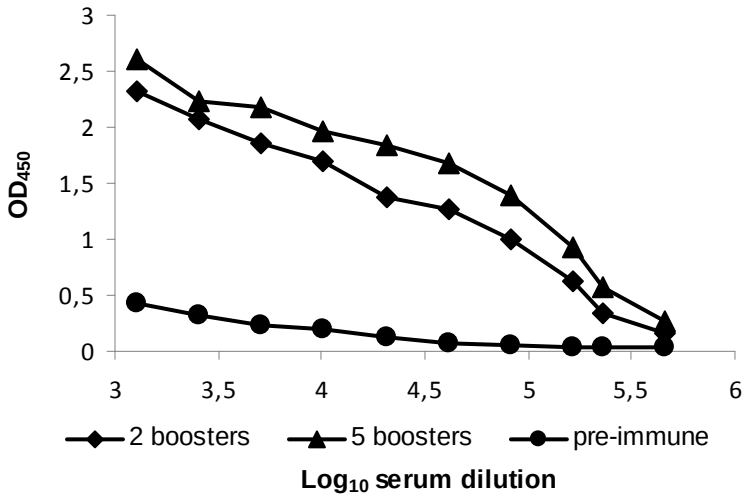
4.1.5 Expression of rHA1 in *pichia pastoris* by high-density cell fermentation

rHA1 was expressed in large scale using high-density cell fermentation. Analysis of rHA1 at 12, 24, 36 and 48 hrs after induction revealed that expression of rHA1 at 28 ℃ for 36 hrs is the best cultural condition for ob taining better expression level. The expression level of rHA1 produced with optimized fermentation process reached 80 mg / L, which is ten-fold higher than the one produced in regular shaking flask. The best method for concentration of rHA1 before purification was with ammonium sulfate 80 % at 4 ℃. Concentration of rHA1 with ammonium sulfate fac ilitates the purification of large volumes of rHA1.

4.2 Immunogenicity of recombinant polypeptides

4.2.1 Immunogenicity of recombinant polypeptides in mice

Vaccination of mice with recombinant polypeptides P1, P2 and rHA1 induced influenza H5 specific antibodies based on recombinant Elisa, whole H5N1 Elisa, as well as IFA. Elisa titres were calculated as half-maximal OD_{450}. Elisa plates coated with the same antigen that was used in vaccination showed higher titres than plates coated with inactivated H5N1 (Figures 10, 11 and table 5). Sera of vaccinated mice were positive by IFA performed on Vero cells infected with H5N1 virus.



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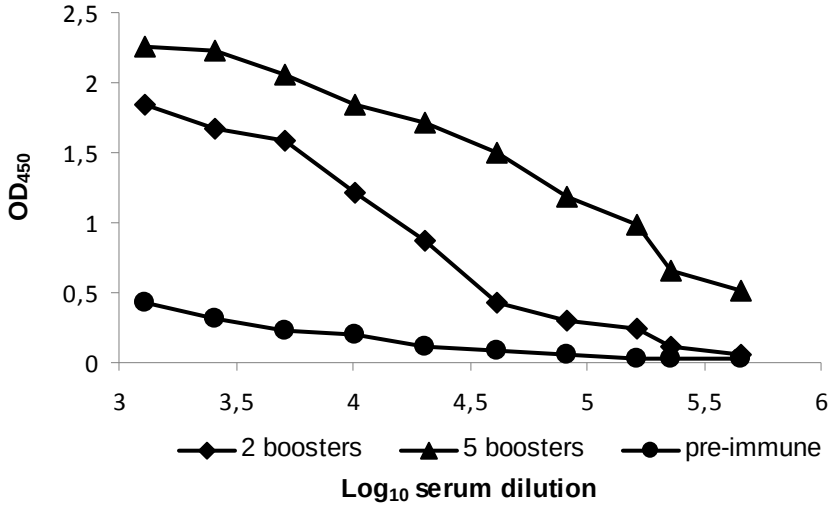

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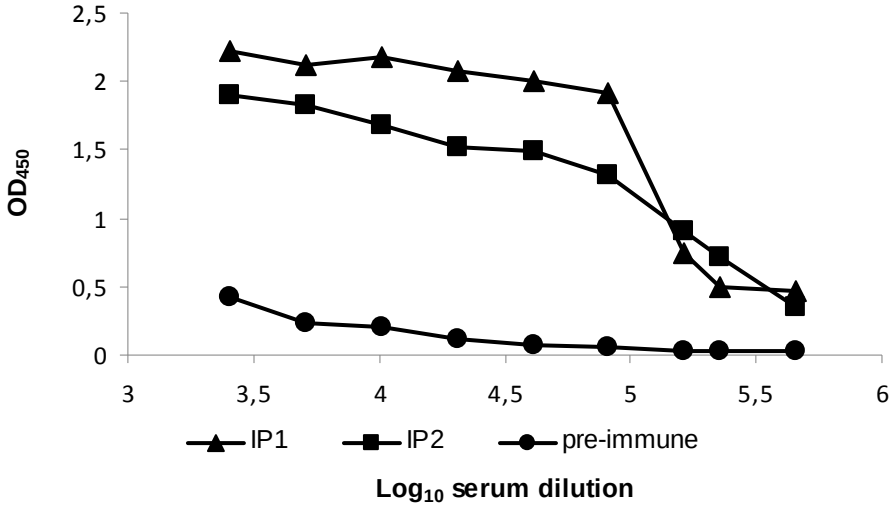


Figure (10): Optical densities (OD_{450}) of mice sera vaccinated with rHA1 polypeptide. Sera dilutions were expressed as reciprocal log_{10}. A: Mice were vaccinated with rHA1 polypeptides using immunization protocols 1 (IP1) Elisa plates were coated with P1 polypeptide (50 ng / well). Elisa titres calculated as half-maximal-OD_{450} were 57517 and 98356 after 2 and 5 boosters, respectively. B: Mice were vaccinated with rHA1 polypeptides using immunization protocols 2 (IP2) Elisa plates were coated with P1 polypeptide (50 ng / well). Elisa titres were 18880 and 103898 after 2 and 5 boosters, respectively. C: Mice were vaccinated with rHA1 polypeptide using IP1 and IP2. Sera were analyzed after 5 boosters with Elisa plates coated with rHA1 polypeptide (50 ng / well). Elisa titres were 140325 and 158622, respectively.

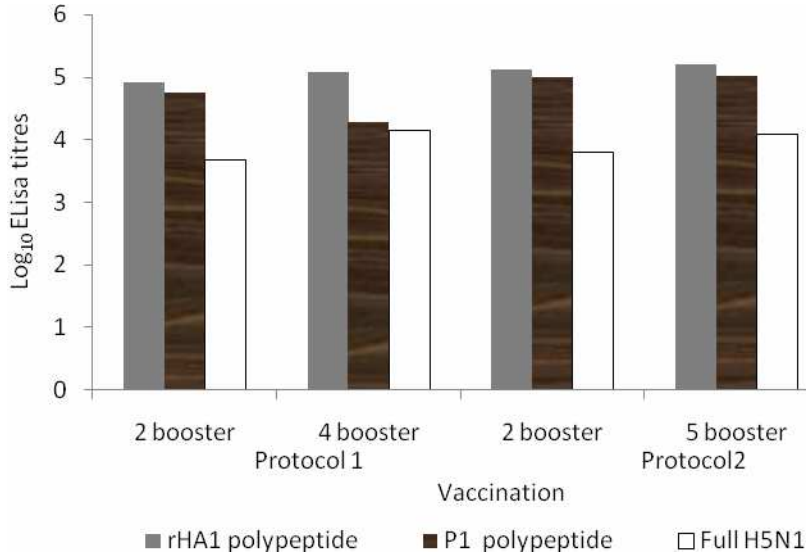


Figure (11): Elisa titres of mice vaccinated with rHA1 polypeptide. Elisa plates were coated with different antigens (rHA1, P1 and whole H5N1). Elisa titres were expressed as reciprocal log_{10}.

Table (5): Immunogenicity of recombinant polypeptides in mice using Elisa

Antigen used in vaccination	Immunization protocol	Elisa titres	
		Homologous Elisa antigen (50 ng / well)	Whole H5N1 Elisa antigen (1 HAU / well)
P1 polypeptide	IP1	12,378	151
	IP2	133,705	2,542
P2 polypeptide	IP1	71,619	2,237
	IP2	>204,800	6,188
rHA1 polypeptide	IP1	140,325	13,935
	IP2	158,622	12,346

IP1 and IP2 are two different immunization protocols (see materials and methods).
Elisa plates were coated with homologous antigen (the same antigen used in vaccination) or whole H5N1 antigen. Titres were calculated as half-maximal $OD_{450.}$

4.2.2 Immunogenicity of recombinant polypeptides in chickens

Immunogenicity of P1, P2, P5 and rHA1 polypeptides was also evaluated in inbred commercial layer chickens in comparison with prepared inactivated H5N1 Thailand virus (3.2.127). Analysis of sera with Elisa showed H5 specific antibodies when Elisa plates coated with the same antigen used in vaccination or with whole inactivated H5N1 (Table 6). Serum samples obtained from chickens vaccinated with P1, P2 and rHA1 were positive by IFA performed on Vero cells infected with H5N1 Thailand isolate. Seroconversion of chickens immunized with P1 and rHA1 polypeptides was significant ($p < 0.0001$) at 4^{th} week post primary vaccination, as analyzed by rElisa (Figure 12.A and B) and IFA (Figure 13). P2 polypeptide induced specific seroconversion at 5^{th} week post primary vaccination. However, P5 polypeptide induced no significant seroconversion at any time of serum analysis. AGID was positive only in chickens vaccinated with inactivated H5N1. Moreover, µNT revealed presence of low neutralizing antibody titres in chickens vaccinated with P1, P2 and rHA1 polypeptides compared with inactivated H5N1 (Table 7). IgY could be detected in egg yolk of chickens immunized with rHA1 and P1 polypeptides at 4^{th} and 5^{th} week post primary vaccination, respectively, as assessed by recombinant Elisa using

homologous antigen that used in vaccination or whole H5N1. Analysis of IgY in egg yolk of chickens vaccinated with rHA1 revealed a lower titres than in serum based on whole H5N1 Elisa at 8 weeks post primary vaccination (Figure 12.C). Egg yolk analysis of chickens vaccinated with P1 and rHA1 polypeptides tested positive by IFA. However, H5 specific antibodies could not be detected in egg yolk of chickens immunized with P2 and P5 polypeptides

Table (6): Results of Elisa in commercial layer chickens vaccinated with recombinant polypeptides

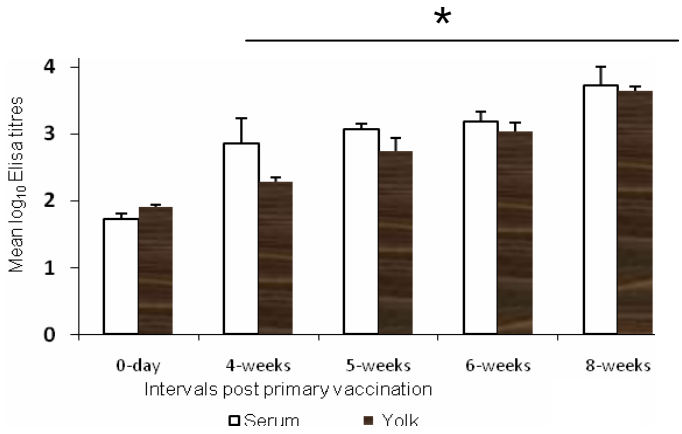
Antigen	**[a]No of chickens**	**[b]Mean Elisa titres (range)**		
		Homologous antigen		**H5N1 antigen**
		6- weeks [c]PV	**8- weeks PV**	**8- weeks PV**
P1	4	205 (152 -319)	1924 (470- 2500)	907 (650- 1200)
P2	4	130 (98- 378)	318 (218- 413)	133 (79 – 213)
P5	4	89 (81- 93)	145 (87- 218)	[d]n.d.
rHA1	4	1059 (418- 1395)	6157 (1220 – 9173)	1840 (1120- 3200)
Inactivated H5N1	4	670 (340- 1212)	2415 (1870-2960)	
Control	4	72 (67 – 87)		84 (83- 89)

[a]Each bird vaccinated with 100 µg of respective polypeptide mixed with 100 ug Gerbu adjuvant. Chickens vaccinated with inactivated H5 received a dose of 8 HAU.

[b]Elisa titres were calculated as half-maximal OD_{450}

[c]PV: Post primary vaccination

[d]Not done.



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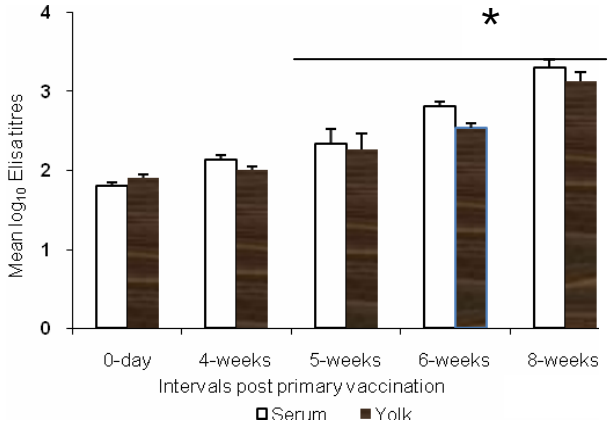

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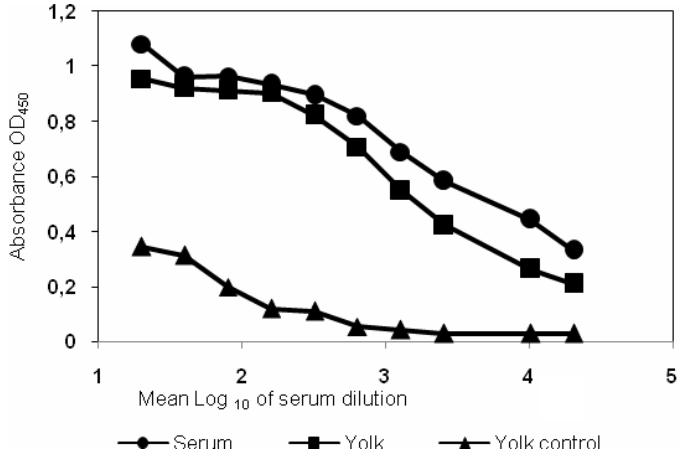


C

Figure (12): Immunogenicity of recombinant polypeptides in commercial layer chickens. A and B): Mean log_{10} Elisa titres of chickens (sera and egg yolk) vaccinated with rHA1, and P1 polypeptides at intervals post primary vaccination, respectively. Elisa plates were coated with homologous antigen used in vaccination. Asterisks (*) indicate significant increase antibody levels compared with negative control. C): Analysis of IgY in serum and egg yolk of chickens immunized with rHA1 at 8 weeks post primary vaccination. Elisa plates were coated with whole H5N1 antigen.

Table (7): Summary results of humoral immune response of chickens vaccinated with recombinant polypeptides

Vaccination	[a]IFA (Positive No. / examined No.)	[b]AGID (Positive No. / examined No.)	[c]µNT
P1	4/4	0/4	16-32
P2	4/4	0/4	2-8
P5	0/4	0/4	[d]n.d.
rHA1	4/4	0/4	32-64
Inactivated H5N1	4/4	4/4	132-264
Control	0/4	0/4	n.d.

[a]IFA was don on Vero cells infected with H5N1 (A/Thailand/1/ Kan-1/2004) virus.

[b]Agar gel immunodiffusion test performed using prepared H5N1 (A/Thailand/1/ Kan-1/2004) antigen.

[c]Microneutralization test, the average OD_{450} was determined for triplicate wells of virus-infected and -uninfected control wells. The endpoint titre was expressed as the reciprocal of the highest dilution of serum with OD_{450} value above the mean + (3 x standard deviation) of the negative control.

[d]Not detected.

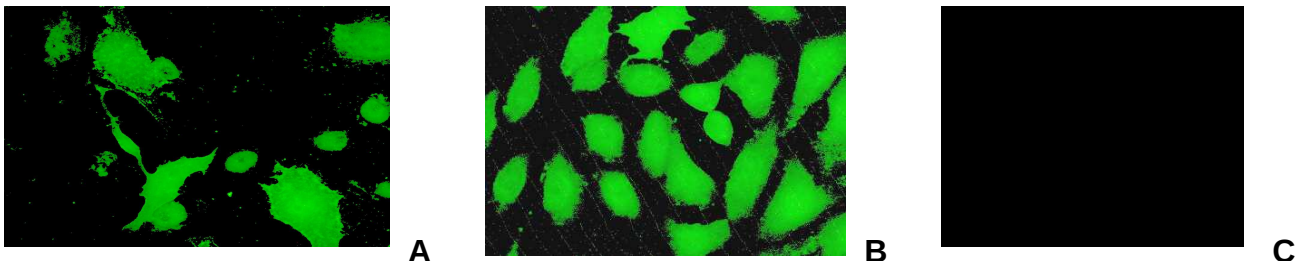


Figure (13): Analysis of chicken serum with IFA performed on Vero cells infected with H5N1 (A / Thailand / 1 / Kan-1 / 2004) virus. A): Sera were obtained from chickens 8 weeks post primary vaccination with rHA1. B): Sera were obtained from chickens 8 weeks post primary vaccination with P1 polypeptide. C: Negative control

4.3 Development of recombinant Elisa for detection of influenza A subtype H5 antibodies

4.3.1 Analysis of serum with HI test

All serum samples (179) were analyzed by HI using commercial homologous (H5N2 (A / chicken / Mexico / 232 / 94 / CPA) antigen. Out of 179 serum samples, 109 were positive by HI. Twenty five of these positive serum samples were retested by HI using heterologous (A / Thailand / 1/ Kan-1 / 2004) H5N1 antigen to be used for determination the validity of rElisa. Haemagglutination inhibiting titres ranged from 4 - 8 $\log_2$ (geometric mean = 6.1) and 3 - 6 $\log_2$ (geometric mean = 4.7) using commercial homologous and hetrologous antigen, respectively (Table 8). All 25 sera obtained from influenza non-vaccinated chickens showed no Haemagglutination inhibiting titres using commercial homologous H5N2 or hetrologous H5N1 Thailand antigens.

Table (8): Distribution of haemagglutination inhibiting antibodies in chickens vaccinated with commercial inactivated H5N2 vaccine using 2 different HA antigens

Antigen	Sera	AI-HI $\log_2$								Geometric
	No	1	2	3	4	5	6	7	8	Mean
[a]Homologous	25				3	6	10	3	3	6.1
[b]Heterologous	25			3	9	5	8			4.7

[a]Commercial H5N2 (A / chicken / Mexico / 232 / 94 / CPA)

[b]H5N1 (A / Thailand / 1 / Kan-1 / 2004) antigen

4.3.2 Reactivity of rHA1 with chicken sera

To study the reactivity of chicken serum samples with rHA1 antigens, twenty five serum samples that proved to be positive by HI using homologous and hetrologous antigen were analyzed by Western blot. Results showed that antibodies of chicken sera were reacted with rHA1 polypeptide. Sensitivity and specificity of Western blot were 100 %. All sera obtained from non vaccinated chicken sera were negative by Western blot (Table 9).

4.3.3 Recombinant Elisa

Twenty five serum samples that proved to be positive by HI using homologous and heterologous antigen as well as by Western blot were analyzed by rHA1 and P1 Elisa. At serum dilution of 1:100 and above, the optical densities of the negative sera plateaued whereas the positive sera continued to show a high optical densities value (Figure 14). Accordingly, serum dilution 1:100 was selected as an optimum dilution to be used in single dilution Elisa. To analyse the validity of recombinant Elisa, these serum samples were tested by single dilution Elisa (using P1, rHA1 and full H5N1 antigens) and the results were compared with HI, Western blot and AGID. Summary results were shown in table (9). The specificities of rHA1-Elisa, rP1-Elisa and whole H5N1 Elisa were 100 %, 72 % and 100 %, respectively, and the sensitivities were 100 %, 80 % and 100 %, respectively. AGID showed low sensitivity (52 %) but high specificity (100 %).

Table (9): Overall sensitivity and specificity for recombinant Elisa compared with other serological tests

Test	Non vaccinated positive No / examined No.	Vaccinated positive No / examined No.	[a]Sensitivity %	[b]Specificity %
[c]rHA1-Elisa	0/25	25/25	100	100
[d]rP1-ELisa	7/25	20/25	80	72
[e]Whole H5N1-Elisa	0/25	25/25	100	100
[f]HI	0/25	25/25	100	100
[g]Wb	0/25	25/25	100	100
[h]AGID	0/25	13/25	52	100

[a]Sensitivity: the probability of correctly identifying true positive (vaccinated).

[b]Specificity: the probability of correctly identifying true-negative (non vaccinated).

[c]Elisa plates were coated with rHA1 polypeptide (50 ng / well).

[d]Elisa plates were coated with P1- polypeptide (50 ng / well).

[e]Elisa plates were coated with whole inactivated H5N1 (A / Thailand / 1 / Kan-1 / 2004) antigen (1HA U/ well).

[f]HI was performed using commercial H5N2 (A / chicken / Mexico / 232 / 94 / CPA) and H5N1 (A / Thailand / 1 / Kan-1 / 2004) antigen.

[g]Western blot was performed using rHA1 polypeptide as antigen.

[h]Agar gel immunodiffusion test was done using H5N1 (A / Thailand / 1 / Kan-1 / 2004) antigen.

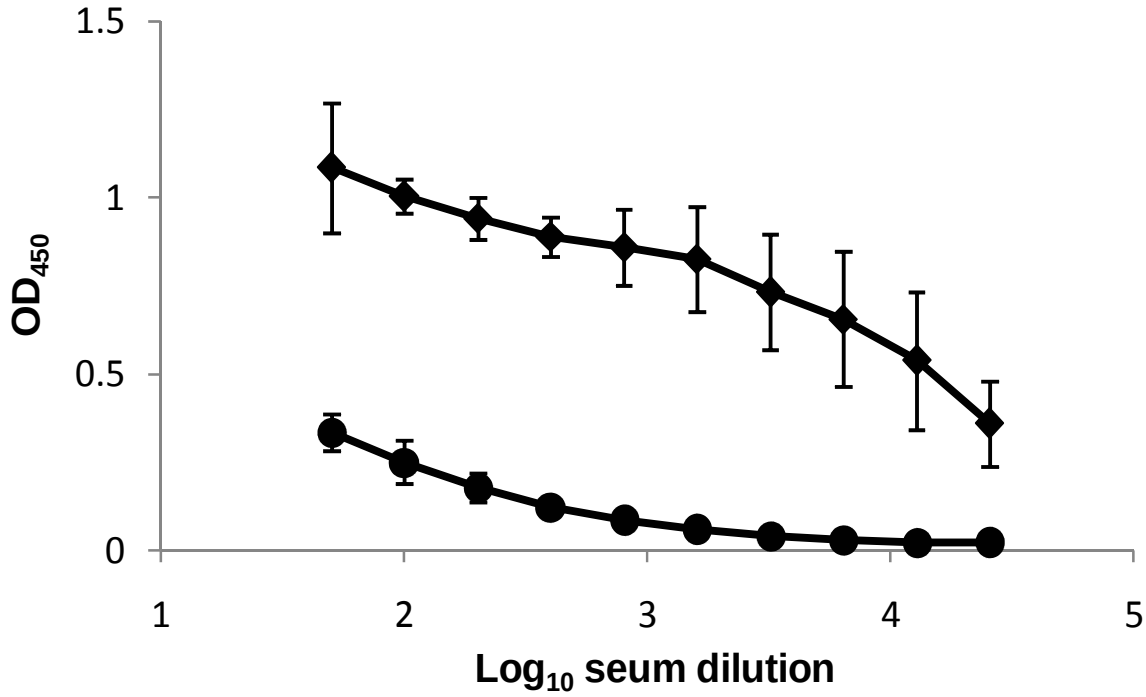


Figure (14): Analysis of chicken sera with rHA1- Elisa. Plates were coated with rHA1 (50 ng / well). Positive sera were obtained from commercial broiler chickens (n = 25) immunized once with commercial inactivated H5N2 vaccine at 7-day-old. Negative sera were obtained from influenza non- vaccinated chickens (n = 25). Serum dilutions were expressed as reciprocal $\log_{10}$.

4.3.4 Agreement between rHA1-Elisa and commercial Elisa and HI

To study the agreement between rHA1-Elisa and cElisa and HI, all serum samples (179) were analysed by rHA1-Elisa, cElisa and HI (using Thailand isolate antigen). Out of 179 serum samples, 109, 139 and 130 positives were obtained by HI test, cElisa and rHA1-Elisa, respectively, (Table 10). The relative sensitivity and specificity between rHA1-Elisa, and cElisa were 93.5 % and 100 %, respectively (table 11). Relative sensitivity and specificity between rHA1-Elisa, and HI was 100 % and 82.8 %, respectively (Tables 12).The agreement ratio between rHA1-Elisa and HI was 84.9 % whereas between cElisa and HI was 76.5 %. Negative serum samples by rHA1-Elisa were confirmed by neutralization test (NT) using H5N1 (A / Thailand / 1/ Kan-1 / 2004) virus.

Table (10): Agreement ratio between rElisa and cElisa and HI

Test	Sera No	Positive	Negative	Positive ratio	Negative ratio
[a]rHA1- Elisa	179	130	49	72.6	27.3
cElisa	179	139	40	77.7	22.3
HI	179	109	70	60.9	39.1

[a]Elisa plates were coated with rHA1 polypeptide 50 ng / well in coating buffer.

[b]Commercial Elisa (Synbiotic Corporation 11011 VIA San Diego, CA 92127).

[c]Haemagglutination inhibition test was done using hetrologous H5N1 (A / Thailand / 1 (Kan-1) / 2004) antigen.

Table (11): Overall relative sensitivity and specificity between rHA1-Elisa, and commercial Flock check Elisa

[a]rHA1 Elisa	[b]c Elisa		Total
	Positive	Negative	
Positive	130 [a]	0[b]	130 [a+b]
Negative	9[c]	40[d]	49 [c+d]
Total	139[a+c]	40[b+d]	179 [a+b+c+d]

[a]Elisa plates were coated with rHA1 polypeptide.

[b]cElisa: Commercial flock check Elisa

Relative sensitivity = a / a + c = 93.5 %. Relative specificity = d / b + d = 100 %.

Table (12): Overall relative sensitivity and specificity between rHA1-Elisa, and HI

[a]rHA1- Elisa	[b]HI		Total
	Positive	Negative	
Positive	109 [a]	12[b]	121 [a+b]
Negative	0[c]	58[d]	58 [c+d]
Total	109[a+c]	70[b+d]	179 [a+b+c+d]

[a]Elisa plates were coated with 50 ng / well rHA1 polypeptide

[b]HI was done using homologous commercial H5N2 (A / chicken / Mexico / 232 / 94 / CPA) antigen.

Relative sensitivity = a / a + c = 100 %. Relative specificity = d / b + d = 82.8 %.

4.5 Validity of rHA1 Elisa for analysis of duck sera

HI analysis of duck sera showed that all samples obtained from ducks vaccinated with commercial inactivated H5N2 showed a Haemagglutination inhibiting titres ranged from 4 log_2 – 8 log_2 (geometric mean 5 log_2) using H5N1 (A / Thailand / 1/ Kan-1 / 2004) antigen. Sera obtained from non- vaccinated ducks showed no Haemagglutination inhibiting antibodies. Analysis of duck sera with rHA1-Elisa showed that rHA1 could react with H5 antibodies in duck sera (Figure 15).

Table (13): Distribution of Haemagglutination inhibiting antibodies in duck vaccinated with commercial inactivated H5N2

Sera	Sera No	AI- HI log_2										GM
		0	1	2	3	3	4	5	6	7	8	
Vaccinated	5						3	1			1	5.0
Non-vaccinated	3	3										0.0

HI was performed using commercial H5N2 (A / chicken Mexico / 232 / 94 / CPA) antigen

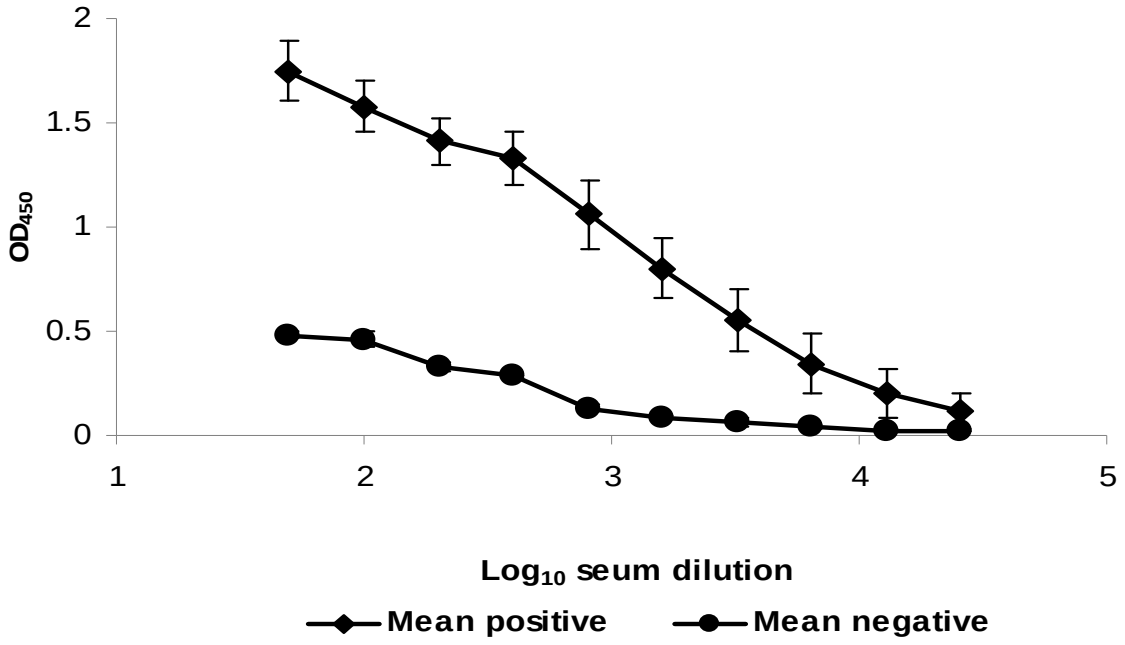


Figure (15): Analysis of duck sera with rHA1-Elisa. Plates were coated with rHA1 (50 ng / well). Positive sera were obtained from ducks (n = 5) immunized 3 times with commercial inactivated H5N2 vaccine. Negative sera were obtained from influenza non- vaccinated ducks (n = 3). Serum dilutions were expressed as reciprocal log_{10}.

5. DISCUSSION

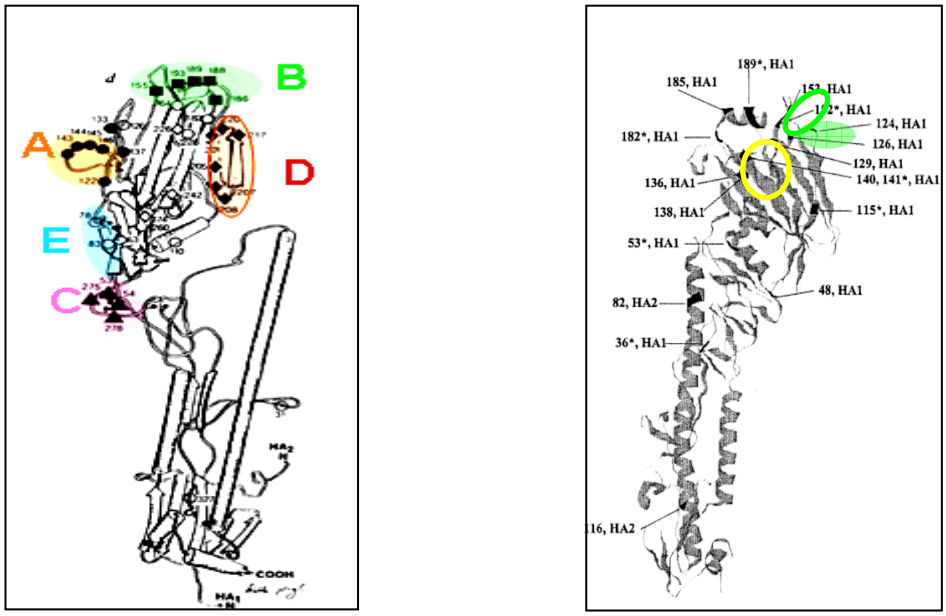
Highly virulent H5 influenza viruses have been isolated from several recent outbreaks in poultry (BAHGAT et al. 2009; BEAN et al. 1985; CAPUA and ALEXANDER 2009; HORIMOTO et al. 1995; TANG et al. 2009). H5N1 influenza virus was transmitted from chickens to humans. Viruses isolated from humans and from birds were very similar in their genetic content and phenotypic features, including virulence for mammals (CLAAS et al. 1998; SUAREZ et al. 1998). Outbreaks of AI in poultry plays an important role in the generation of pandemic viruses for humans (CAPUA and ALEXANDER 2007; CAUTHEN et al. 2000; LAHARIYA et al. 2006). Emergency vaccination for AI has become an acceptable tool, in conjunction with other measures, for combating the spread of AI. Using emergency vaccination to reduce the transmission rate could provide an alternative to pre-emptive culling to reduce the susceptibility of healthy flocks at risk. The effectiveness of such program depends on variables such as the density of poultry flocks in the area, level of biosecurity and its integration into the industry, characteristics of the virus strain involved, and practical and logistical issues such as vaccine availability and adequate and speedy administration (CAPUA et al. 2009). The traditional egg-based vaccines have been successfully used for more than 50 years to prevent influenza. They are reliable, effective (if there is a good match), and affordable. However, the production cycle of the egg-based vaccines is lengthy and heavily dependent on egg supply and unable to be developed quickly in response to the urgent need in an influenza pandemic (COX 2005; OSTERHOLM 2005). To meet the challenge of a potential influenza pandemic, however, a reliable expression system and a quick, efficient downstream purification process are needed. In the present study, truncated sequences were expressed in *Pichia pastoris* to be used in vaccination and diagnostic purposes.

Immunogenic regions of H5 influenza

HA, a homotrimeric class I membrane glycoprotein, is quantitatively the major surface protein of influenza virus and the major antigen against which neutralizing antibodies are elicited. Therefore, recombinant HA is favourable antigen as a candidate influenza vaccine. HA mediates the attachment of the virus to the target cell through specific binding with sialic acid-containing determinants and, following internalization, the release of the viral content into the attacked cell (WHITE et al. 1982; WILEY and SKEHEL 1987). HA-specific antibodies are protective as a result of their ability to prevent virus attachment and penetration of the host cell, and presumably by

interfering with the low-pH-induced conformational change of the HA molecule needed for fusion (KIDA et al. 1982; KIDA et al. 1985). Because of the immune selection pressure, HA is the viral component which is most important in antigenic drift. The HA monomer is synthesized as a single polypeptide chain which undergoes post-translational cleavage at two sites: the N-terminal signal sequence is removed and, depending on the host cell and virus strain, the molecule is cleaved, with the removal of one or more intervening residues, resulting in two polypeptide chains called HA1 (36 kDa) and HA2 (27 kDa), linked via a disulfide bridge (SINGH et al. 1990; SKEHEL et al. 1982). A c-terminal stretch of hydrophobic amino acids anchors HA to the viral membrane and, though not essential for secretion, this sequence plays a major role in the trimerization process (SINGH et al. 1990). The immunogenic potential of yeast derived HA may be appropriate for the development of an easily adaptable, safe and economic alternative to the currently used influenza vaccines. Furthermore being a recombinant expression system, it may be possible to improve its protective properties by genetic engineering. Recombinant protein vaccine was found to be a feasible approach to a variety of pathogens in poultry for improvement of new vaccines; i.e IBD (OMAR et al. 2006), IB (YANG et al. 2009), Coccidiosis (DING et al. 2008; LILLEHOJ et al. 2000), ND (LEE et al. 2010), Runting Stunting Syndrome (SELLERS et al. 2010), Reo virus (WU et al. 2009), AI (LIN et al. 2008; XIE et al. 2009). Peptide vaccination has many advantages and could be an alternative vaccine to commercially available vaccines. Subunit vaccines based on conserved antigens provide broader protection (TOMPKINS et al. 2007). The influenza HA glycoprotein is the primary target of neutralizing antibodies (Wiley et al., 1981). The H3 structure was initially used to characterize the antigenic structure of H5 (PHILPOTT et al. 1990). In this study truncated HA polypeptides were expressed to analyze their potential use in vaccination and for diagnostic purposes. Five neutralizing epitopes were identified (designated A-E) and their location was mapped on the three-dimensional model of the H3 HA molecule (WILEY et al. 1981). The sequence and three-dimensional structure of the HA antigenic epitopes has been characterized in detail only in the H3 subtype of influenza A (WILEY et al. 1981). The H3 three dimensional model has since been used in studies of the H1 subtype (CATON et al. 1982)**,** the H2 subtype (TSUCHIYA et al. 2001) and, to a limited extent, the H5 subtype (PHILPOTT et al. 1990)**.** The region 136-141 corresponds to site A in the H3 structure (140±145 in H3 numbering) and to site Ca2 in H1 (CATON

et al. 1982), forming a loop at the side of the HA molecule. One amino acid change in this region (position 145, H3 numbering) was demonstrated in an H5 escape mutant by (PHILPOTT et al. 1990). The amino acid changes at positions 152 and 153 (156 and 157 in H3 numbering) correspond to the area involved in the formation of site B in the H3 molecule (PHILPOTT et al. 1990) showed a change in the H5 molecule at position 156 (H3 numbering). The H5 area 124-129, which corresponds to 129-133 in the H3 sequence, is located outside any site in the H3 HA structure recognized by virus-neutralizing mAbs (WILSON et al. 1981b) but partially overlaps a region involved in the antigenic site Sa in H1 HA (CATON et al. 1982).



A **B**

Figure (16): Structural identification of the antibody- binding sites of influenza H3 and H5 according to WILSON et al. (1981b) and KAVERIN et al. (2002), respectively.
A): HA contains 5 antigenic sites designated A (140-146), B (187-196 and 155-160), C (275- 278 and 53 - 54), D-(201-202), and E (60-83).
B): Structural identification of the antibody- binding sites of influenza H5. Site A is a conformational epitope but not conserved in H5 (136- 141) and 140- 145). Site B is described as a discontinuous epitope (152- 153) and 156-157 and 124-129 and 129-133.

In this study, four regions were chosen for production of recombinant polypeptides and designated P1, P2, P5 and rHA1 (Table 1 and Figure 17).

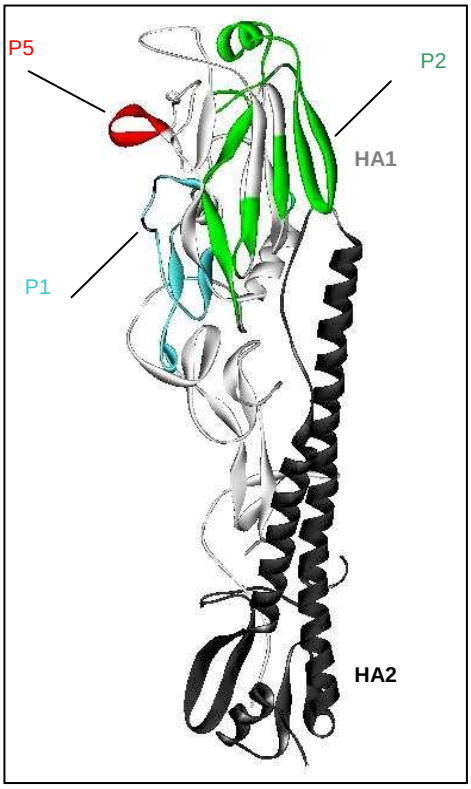


Figure (17): Crystal structure of influenza A subtype H5 virus monomer (1JSM.pdb). The location of coding sequences used for expression within HA1 was coloured with WebLab ViewerLite program.

P1 polypeptide, corresponds to the neutralizing epitope of Site E of H3 (WILEY et al. 1981). Alignment of H5 revealed that this sequence is conserved in H5 viruses (40 amino acids residues long). It is worthy to mention that H5 site that is equivalent to site B of H5 appears to be more complex in H5 than in H3. Site B in H5N2 (A / Mallard / Pennsylvania / 10218 / 84) contains not only the region present in H3 site B but also the region 124- 129 (KAVERIN et al. 2002), which partially overlaps site Sa of H1 (CATON et al. 1982). Accordingly, P2 epitope was chosen as it consists of the receptor binding site, site D and parts of site B, conserved in H5, 97 amino acid residues long, contains a glycosylation site. P5 is a conformational epitope in H5 (KAVERIN et al. 2002), not conserved, 30 amino acid residues long and contains a glycosylation site. Also, rHA1 which contains the majority of antigenic determinants

are responsible for generation of virus-neutralizing antibodies, 320 amino acid residues long and contains 5 glycolsyation sites.

***P. pastoris* expression**

Recombinant HA proteins can be produced in different ways such expression in insect cell system (JOHANSSON 1999; LAVER and WEBSTER 1976; POWERS et al. 1995; TREANOR et al. 2001; 2006) or in the recombinant baculovirus expression system in insect larvae (SUGIURA et al. 2001). Previous baculovirus / insect cell systems have been used to express HA genes isolated from AI subtypes. However, a protein band corresponding to rHA1 from baculovirus infected cells was not observed by SDS-PAGE of total cell protein. This could have been due to a low level of expression or alternatively incorrect glycosylation of the polypeptide in insect cells or toxicity of insect cells (POSSEE 1986). HA1 was expressed in monolayer or suspension culture insect cells by infection with the recombinant baculovirus (NWE et al. 2006). Although *E. coli* expression system is not complicated and high amount of recombinant protein could be produced when comparing to other production system, *E. coli* often leads to production of the expressed proteins in insoluble inclusion bodies (TSUMOTO et al. 2003). Accumulation of expressed foreign protein in *E. coli* in the discrete form of the inclusion bodies is the greatest drawback of bacterial expression system (MARSTON. 1986). *P. pastoris* has the potential of high-level expression and rapid growth to very high cell densities in inexpensive media (ROMANOS et al. 1992). In addition, *P. pastoris* is a highly successful system for the production of a variety of heterologous proteins. Choosing of this particular expression system can be attributed to several factors. *P. pastoris* has the ability to produce foreign proteins at high levels, either intracellular or extracellular. In addition *P. pastoris* has the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing. Moreover, *P. pastoris* system strong promoters are available to drive the expression of a foreign gene(s) of interest thus enabling production of large amounts of the target protein(s) with a relative lower cost than most other eukaryotic systems (CEREGHINO et al. 2002; DALY and HEARN 2005). Yeast is the favored alternative host for expression of foreign proteins in research, industrial or medical use (HITZEMAN et al. 1981; WEIDNER et al. 2010). As a food organism, it is highly acceptable for the production of pharmaceutical proteins. Additional advantages of *P. pastoris* are the availability of complete genome sequences, the stable high copy

numbers of nuclear plasmids and ability to secrete the target protein (HITZEMAN et al. 1990). Accordingly, *P. pastoris* was chosen as an expression system to be used in this study. Coding DNA fragments of full length or epitope-based truncated sequences of influenza A subtype H5N1 (A / Thailand / 1 (Kan-1) / 2004) were cloned in to pAOX vector for recombinant production using gene specific primers. Appropriate expression cassettes were used for transformation of *P. pastoris* cells (strains GS115, SMD1168H). *E- coli* provide a well defined simple system for stable storage of the construct as well as for isolation of large quantities for verification of the inserted sequence. Accordingly, before transformation to yeast genome, pAOX plasmids containing inserts were transformed into XL10®-Gold ultracompetent *E-coli* cells and plasmids were isolated for analysis. Plasmids were subjected to double digestion with restriction enzymes. For further analysis, the gene of interest was sequenced. Transformation is a crucial step in hetrologous protein expression barrier such as cell walls and cell membrane restrict effective uptake of foreign DNA. Moreover the expression cassette has to integrate by homologous recombination, resulting eventually in stable transfromants (CEREGHINO et al. 2002; ORR-WEAVER et al. 1981). To study several clones for their protein production capacity, it is necessary to obtain sufficient large numbers of transformants. The pAOXα vector integrates at the AOX1 site of the *Pichia* genome. With the developed improved transformation protocol described in by Dr. Kathrin Rall (Virology institute, Leipzig University), coding DNA sequences of P1, P2, P5 and rHA1 were cloned in frame downstream of the alpha factor leader into pAOXα. The insert length varied from 159 to 1007 bp. In our transformation protocol many colonies of transformed cells arose either GS115 or SMD1168H. Our own experience shows that not all transformed clones express the desired peptide or protein at high levels. Consistently, several clones completely failed in recombinant protein production and growth of such clones is not helpful. Selected clones were used for secretory expression of polypeptides fused to his-tag facilitating detection in culture supernatants using Western blot. The four developed polypeptides were identified by SDS-PAGE followed by Western blot in both cell lysate and culture supernatant. Secretion requires the presence of a signal sequence on the foreign protein to target it to the secretory pathway. While several different secretion signal sequences have been used including the native secretion signal present on some heterologous proteins, success has been variable. However the secretion sequences from *S. cervisae* factor PrePro peptide have been

used with the most success. *S. cervisae* factor prepro peptide consists of a 19 aa signal pre-sequence followed by a 66- residue pro sequence (KURJAN and HERSKOWITZ 1982). Signal processing starts with the removal of the pre signal by a signal peptidase in the endoplasmic reticulum followed by cleavage of the pro leader sequence between aa Arginine and Lysine by kex2 endopeptidase. Finally Glu-Ala repeats are cleaved by ste13 protein (BRAKE et al. 1983). The close proximity proline residues can influence cleavage efficiencies of Kex2 and Ste3 proteins and the tertiary structure formed by a foreign protein may protect cleavage sites from these proteases.

Glycosylation analysis

P. pastoris has the potential of performing post-translational modifications including N-glycosylation. It begins in the endoplasmic reticulum (ER) with the transfer of a lipid-linked oligosaccharide unit Glc3Man9GlcNAc2 (Glc: Glucose GlcNAc: N. acetylglucosamine) to Asparagine Asn-Xser / Thr. (CEREGHINO et al. 2002; DALY and HEARN 2005). Analysis of the *N*-linked carbohydrates showed the presence, predominantly, of (*N*-acetylglucosamine)$_2$ Man$_{8–10}$ residues (SAELENS et al. 1999). This result is in agreement with the reported average 8–14 mannose residues added post-translationally by *P. pastoris* residues (SAELENS et al. 1999) and is in striking contrast with the observation of the rather exceptional hyperglycosylated nature of soluble recombinant neuraminidase containing *N*-glycans with 30–40 mannose residues, from the same organism (MARTINET et al. 1997). Although the molecular mechanisms determining the outcome of the glycosylation pattern of a glycoprotein in a particular eukaryotic host organism remain enigmatic, one might speculate that the folding kinetics play a role. Glycoproteins that spend longer in the early exocytic vesicles might be more susceptible as a substrate for glycosyltransferase activity. Recognition of *P. pastoris*-secreted HA0s by a panel of mAbs implies that at least part of the molecule is correctly folded (SAELENS et al. 1999).

In this study, rHA1 has a theoretical molecular mass of 39.67 kDa but when the protein was expressed in *P. pastoris* it gave a broad smear above the expected size as analyzed by SDS-PAGE and Western blot. Endo H$_f$ is a recombinant protein fusion of Endoglycosidase H and maltose binding protein. Endo H$_f$ cleaves within chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoprotein (ESHAGHI et al. 2005)**.** It is used to get information about carbohydrate groups attached to glycoproteins and glycopeptides. After removal of mannose

residues, the proteins were analyzed using SDS-PAGE and Western blot. Treatment of purified rHA1 with Endo H_f revealed that its aberrant migration resulted from post-translational glycosylation. Although the exact structure of the oligosaccharides did not analyzed, the most commonly observed N-linked glycans in *P. pastoris* secreted recombinant protein are short Man8GlcNAC and Man9 GLCNAc (MONTESINO et al. 1998). The N-glycosylation appears to be important in correct protein folding, conformational stability and resistance to protease degradation during synthesis (ZHU et al. 1998)**.** However, the site of glycosylation should be determined carefully; it should not interfere with the folding of the protein and should not cover the active site of the molecule (SAGT et al. 2000). N glycosylation containing only the first residue, GlcNAc, would be sufficient to maintain the conformational stability (ERBEL et al., 1999; ERBEL et al., 2000; WELLER et al. 1996; WILSON et al. 1981a). It is worthy to mention that lectins are extremely useful tools for the investigation of carbohydrates on cell surfaces as well as for the isolation and characterization of glycoproteins. Numerous lectins have been isolated from plants as well as microorganisms and animals (SHARON and LIS 2004). Lectins bind principally to oligosaccharides and cell surface glycoproteins and glycolipids that contain appropriately linked mannose residues (CHAN and REES 1975; GRODECKA et al. 2010; SAINZ-PASTOR et al. 2006). rHA1 could be analyzed by blotting with concanavalin A. Analysis of P2 polypeptide by Western blot showed multiple bands due to different glycosylation pattern as it contains a glycosylation site. Analysis of culture supernatant of P5 by Western blot revealed that P5 polypeptide is secreted in a very low amount (detected only after concentration by ultraconcentration). Analysis of P5 polypeptide in cell lysate by SDS-PAGE and Western blot showed a size of about 21 kDa. This is attributed to the glycosylation of alpha factor. The pro sequence of alpha factor contains three N-linked- glycosylation sites and a dibasic-kex2–endopeptidase. P5 polypeptide could not bind with Ni-NTA affinity chromatography either under natural or denaturing condition. As optimal purification using Ni-NTA is dependent on the amount of 6x his-tagged protein, possibly, P5 polypeptide hist-tag in the N-terminal might be removed by proteolysis. Eshaghi and others (ESHAGHI et al. 2005) mentioned that His-tag in N terminal of expressed proteins was suspected to proteolytic removal in sf-9 cells. P2 polypeptide contains one glycosylation site and its theoretical mass is ~14 Kda, however, analysis by SDS-PAGE and Western blot resulted two bands (about 10 and ~22 kDa). Presence

of multiple bands is attributed to not all the protein glycosylated. Endo H_f could not remove the glycosylation residues of P2 and P5 polypeptide. Furthermore, both P2 and P5 polypeptides did not react with concanavalin A. Posttranslational modifications are also important in determining the efficacy of secretion of a protein, since the overall fold will affect the processing of the signal sequence. Expression of rHA1 in the presence of tunicamycin, was lead to partial deglycosylation but decrease in the amount rHA1 secreted in supernatant.

Immunogenicity of recombinant polypeptides

The need for vaccination of poultry is highly controversial. Anyway, speculation about potential problems of vaccine use must be balanced with the real problem of outbreaks in susceptible poultry. Vaccination of chickens against HPAI must be considered complement to other control measures. Vaccination of chickens with recombinant polypeptides P1, P2 and rHA1 showed H5 subtype specific antibodies as analyzed with Elisa, and IFA. Elisa titres were lower than that obtained in immunized mice which indicate that the vaccination regime in chickens should be optimized. The µNT revealed presence of neutralizing antibodies in chickens vaccinated with P1, P2 and rHA1 but with low titres as compared with inactivated vaccine. At present time we can not determine the reason for the low neutralizing activity of our polypeptides. Several reports (CHIU et al. 2009; PORTOCARRERO et al. 2008; SPITSIN et al. 2009; TREANOR et al. 2006; WEI et al. 2008) mentioned that selection of virus strain (s) and / or the epitope of HA polypeptides, expression system, choice of adjuvant, dosage, peptide folding may have an impact on the ability of HA to generate a protective antibody response. However, rHA1 polypeptide purification required denaturation which might be the reason for low neutralizing activity, hence, in a recent study (CHIU et al. 2009) it was found that the best method for generating HA1-specific neutralization determinant is on-column oxidative refolding procedures with Glutathione. On the other hand the effect of glycosylation of our developed polypeptides on the immune response should be studied in details. Glycolsyation may change the function and characteristics of the recombinant protein (KREIJTZ et al. 2007). It was mentioned that removal of structurally non essential glycans on viral surface glycoproteins may be a very effective approach for vaccine design against influenza and other human viruses (WEI et al. 2008). HA glycosylation affects the function of influenza HA (WAGNER et al. 2002). Interestingly, as the level of glycosylation on influenza H3N2 has increased since 1968, the morbidity,

mortality, and viral lung titres have decreased (VIGERUST et al. 2007). HA with a single GlcNAc attached to the glycosylation sites showed relaxed specificity but enhanced affinity to α 2,3 sialosides suggests that the N-glycans on HA may cause steric hindrance near the HA–receptor binding domain. The high specificity for receptor sialosides may prevent the virus from binding to some other specific glycans on the human lung epithelial cell surface. On the other hand, HA with truncated glycans can recognize α 2, 3 receptor sialosides with higher binding affinity and less specificity, suggesting that reducing the length of glycans on HA may increase the risk of avian flu infection. It is, however, unclear how the changes of HA–receptor interaction via glycosylation affect the infectivity of the virus and the NA activity in the viral life cycle. HA with a single GlcNAc is a promising candidate for influenza vaccine because such a construct retains the intact structure of HA and can be easily prepared (e.g., via yeast). It also can expose conserved epitope hidden by large glycans to elicit an immune response that recognizes HA variants in higher titre (SUI et al. 2009). This strategy opens a new direction for vaccine design and, together with other different vaccine strategies and recent discoveries of HA neutralizing antibodies should facilitate the development of vaccines against viruses such as influenza, hepatitis C virus, and HIV (EKIERT et al. 2009; HOFFMANN et al. 2005; HULEATT et al. 2008; KASHYAP et al. 2008; SCANLAN et al. 2007; SCHEID et al. 2009; STEVENS et al. 2006; SUI et al. 2009; YANG et al. 2007). Glycans near antigenic peptide epitopes interfere with antibody recognition (OHUCHI et al. 1997) and glycans near the proteolytic activation site of HA modulate cleavage and influence the infectivity of influenza virus (DESHPANDE et al. 1987)**.** Mutational deletion of HA glycosylation sites can affect viral receptor binding (GUNTHER et al. 1993)**.** However, little is known regarding how the structure and composition of its glycans affect HA activity, including structure, receptor binding, and immune response. The use of immunogenic peptides has been proposed as a means of developing defined vaccines. Once a potentially protective peptide has been identified, it must be delivered to the immune system in a form which elicits anti-peptide antibodies that will recognize and neutralize the infectious agent. As a consequence of the small size of P1 (7.5 kDa), P2 (13.9 kDa) and P5 (6.5 kDa), it has been suggested that peptides require chemical coupling to a carrier as tetanus toxoid, to enhance their immunogenicity. But, chemically coupling short peptides to carrier proteins can result in poor immunogenicity. However, the immunogenicity

could be dramatically improved by synthesizing peptides consisting of tandem repeats of the epitope (BROEKHUIJSEN et al. 1987; DIMARCHI et al. 1986). AGID was positive in chickens immunized with inactivated H5 and negative in chickens vaccinated with recombinant polypeptides indicating that the peptides could not elicit immune response towards precipitating antibodies, this in accord with (SNYDER et al. 1985; SUAREZ 2005) who mentioned that AGID test targets M protein and NP and subunit vaccines did not elicit immune response towards these proteins and this could help in DIVA. But the sensitivity of AGID should be considered as this test has high sensitivity but low specificity. Four DIVA strategies have been proposed for AI to overcome this limitation. All four DIVA strategies have advantages and disadvantages, and further testing is needed to identify the best strategy to make vaccination a more viable option for avian influenza. The most common is the use of unvaccinated sentinels. The main disadvantage is in the management of the sentinel birds, because they must either be marked or caged separately from the other birds in the house. There is also concern that these naıve birds may increase the risk of infection of the flock (SUAREZ 2005)**.** A second approach is the use of subunit vaccines targeted to the HA protein that allows serologic surveillance to the internal proteins. Because antibodies to the HA and neuraminidase proteins provide the primary protection against avian influenza virus challenge, it is possible to protect birds by having only these proteins in a vaccine. Antibodies to the HA protein in particular are critical for protection, and many experimental subunit vaccines have included only the HA protein. Many different types of subunit vaccines, including virus vectored vaccines and vaccines using protein expressed in different culture systems, have been shown to provide protection from HPAI challenge. However, only the fowl pox-vectored recombinant vaccine for the H5 subtype is available commercially (LEE et al. 2006). The subunit vaccines provide the most flexibility to work with existing type A serologic surveillance tests, specifically the AGID and commercial ELISA tests that target the M or NP structural proteins. Vaccinated birds will not develop antibodies to the internal proteins, providing a clear distinction between infected (has antibodies to HA, M and NP) and vaccinated birds (has antibodies to HA but no antibodies for M or NP). As previously mentioned, the only commercially available subunit vaccine for AIV is the fowl pox recombinant vaccine, and this vaccine is only available for the H5 subtype. This vaccine contains the A/Turkey/Ireland/83 H5 HA gene, and experimentally this vaccine has been shown to be protective for many

HPAI viruses (SWAYNE and SUAREZ 2000; TAYLOR et al. 1988). A third strategy is to vaccinate with a homologous HA to the circulating field strain, but a hetrologous neuraminidase subtype. Serologic surveillance can then be performed for the homologous NA subtype as evidence of natural infection (CAPUA et al. 2003). The fourth strategy is to measure the serologic response to the non-structural protein 1 (NS1). This DIVA approach was demonstrated previously with equine influenza viruses in horses using an Elisa format (BIRCH-MACHIN et al. 1997). Experimentally, the approach also works with chickens with purified killed vaccines. However, commercial AI vaccines are made with allantoic fluid from infected embryonating chicken eggs and are only partially purified. Therefore, they contain small amounts of NS1 protein in the vaccine as a contaminant from the lysed cells in the allantoic fluid, and vaccinated chickens will develop some antibodies to the NS1 protein, particularly after repeated vaccinations. This small amount of NS1 antibody does make it more difficult to use the NS1 DIVA strategy, but infected birds appear to have higher levels of antibody as compared to vaccinated birds. By diluting the sera before testing, a clear distinction, at least experimentally, can be made between vaccinated and infected birds (TUMPEY et al. 2005). Our recombinant polypeptides could not elicit immune response towards precipitating antibodies. Accordingly, Elisa coated with internal proteins and AGID test could be used as DIVA tools. It is worthy to mention that the molecular mass of the antigen was an important factor in eliciting antibodies in hens as reflected in the titre of the antibodies in their yolk. Antigens with molecular mass equal to or higher than that of human IgG appear to produce a good responses in hens whereas antigens of lower molecular mass and less appear to be poor antigen (POLSON et al. 1980). The tendency of antigenic response of hens on molecular size of the antigen is not unique but is a well-known phenomenon which is frequently observed when mammalian species were hyperimmunized with antigens of low molecular weight. The only difference being that the responses of low molecular weight is poorer in hens (POLSON et al. 1980). In addition, there are 3 classes of antibodies in chickens, namely IgY, IgA, and IgM. Chicken IgA and IgM are similar to mammalian IgA and IgM in terms of molecular weight, structure, and immunoelectrophoretic mobility (LILLEHOJ et al. 2000). Although structural differences exist between IgY and mammalian IgG, IgY is considered the avian equivalent to mammalian IgG. In eggs, IgY is present predominantly in the egg yolk (LILLEHOJ et al. 2000), whereas IgA and IgM are present in the egg white as a result

of mucosal secretion in the oviduct (ROSE et al. 1974)**.** In chickens, the transfer of IgY from the dam to her offspring takes place in a 2-step process. In the first step, IgY is taken up into the egg yolk by the IgY receptors on the ovarian follicle from the dam's blood (LOEKEN and ROTH 1983). In the second step, IgY is transferred from the egg yolk to the offspring via the embryonic circulation. Yolk IgY is transported at a low rate across the yolk sac into the embryonic circulation as early as embryonic day 7 (KRAMER and CHO 1970). The rate of transfer started to increase by embryonic day 14 and by embryonic day 19 to 21, there was a steep rise in the rate of transfer of IgY from the egg yolk to the embryonic circulation (KOWALCZYK et al. 1985). The amount of IgY transferred to the egg yolk has been reported to be proportional to maternal serum IgY concentrations (AL-NATOUR et al. 2004; LOEKEN and ROTH 1983). As reported by Kaspers and coworkers (KASPERS et al. 1991)**,** maternal antibodies to AI should be considered. IgY was analysed in egg yolk of chickens immunized with our recombinant polypeptides. In the present study, the amount of IgY of chicken, immunized with P1 and rHA1, transferred to the egg yolk has been reported to be proportional to maternal serum IgY concentrations which is in accordance with (Al-NATOUR et al. 2004; LOEKEN and ROTH. 1983). However, no detectable antibodies in egg yolk of chickens immunized with P2 and P5. This may attributed to their low immunogenicity.

The Use of recombinant polypeptides to improve a diagnostic method for AI

The use of live culture Elisa, and HI assay has biosafety implications (STEPHENSON et al. 2009) and the solution to these problems is to use a standardized recombinant antigen created using recombinant technology (WANG et al. 2010). Conventional subtype-specific methods for serological investigations as HI, and NT have significant limitations (PRABAKARAN et al. 2009). Other assays, such as complement fixation, neuraminidase-inhibition test or microneutralization assay require special equipments and complex procedures. The indirect Elisa (I-Elisa) using crude or purified viral antigen on the solid-phase to detect viral specific antibodies has been developed for detection of chicken and turkey antibodies to AIV (ABRAHAM et al. 1988; ADAIR et al. 1989). The recombinant AI NP expressed in *E- coli* was purified, coupled with latex beads, and used as an antigen for the latex agglutination test (LAT) test. LAT test proved to be useful for monitoring AIV infection in the field (HORIE et al. 2009). Serologic testing of wild birds for AIV surveillance poses problems due to species differences and nonspecific inhibitors that may be present in sera of wild birds. Two

commercial competitive Elisas detect AI antibodies in experimentally infected partridges, whereas HI was negative (ZHOU et al 1998). Both Elisas detected AIV-antibody-positive samples were negative by specific HI against 9 of the 16 existing HA subtypes. Presumably this may reflects higher sensitivity of competitive ELISA when compared to HI. A competitive Elisa was developed as a serologic diagnostic tool to detect antibodies against NA subtype 3 of AIV. The NA antigen used in this ELISA was obtained by pronase treatment of allantoic fluid of specific-pathogen-free (SPF) eggs infected with AIV. The NA specific monoclonal antibodies were produced from purified NA. N3 c-ELISA could detect the antibodies from SPF chickens or commercial chickens vaccinated with H9N3 subtype of AIV. The sensitivity and specificity of the N3 c-ELISA were 83.7 % and 95.6 %, respectively (KIM et al. 2010)**.** A recombinant HA of A / Vietnam / 1203 / 04 (H5N1) was expressed in mammalian cells, purified, and used for generation of H5-specific monoclonal antibodies (MAb). The purified H5-Bac was used to develop a competitive Elisa to detect H5 antibodies. Comparison of the results of the competitive Elisa with results obtained by HI showed a gradient of the sensitivity (turkeys > ducks > chicken). The described results showed that H5-specific antibodies in sera can be detected in a species-independent approach by using a recombinant protein (DLUGOLENSKI et al. 2010). An indirect ELISA was developed using baculovirus, purified, recombinant N1 protein from A / chicken / Indonesia / PA7 / 2003 (H5N1) virus. The N1-ELISA showed high selectivity for detection of N1 antibodies, with no cross-reactivity with other neuraminidase subtypes, and broad reactivity with sera to N1 subtype isolates from North American and Eurasian lineages. N1-ELISA can facilitate a vaccination strategy with differentiation of infected from vaccinated animals using a NA hetrologous approach (LIU et al. 2010). Because the NS1 is expressed in influenza virus-infected cells, and it is not packaged in the virion, it is an attractive candidate for a DIVA differential diagnostic test (AVELLANEDA et al. 2010). Active surveillance for AIV has expanded from chicken to various poultry species including duck. An alternative to serum, antibody monitoring of laying breeder duck using egg yolk with competitive ELISA is feasible and is recommended (JEONG et al. 2010). In addition, the results reflect the necessity of validation Elisa for individual species or at least families (PEREZ-RAMIREZ et al. 2010). The use of chicken egg yolk antibody as an alternative to serum has shown a high degree of correlation among AGID, HI and ELISA approaches (BECK et al. 2003). Detection of NP, N3 and N7 antibodies to AI virus by

indirect ELISA using yeast-expressed antigens revealed that these indirect Elisas are rapid, sensitive, specific and can be used as promising tests during serological surveillance (UPADHYAY et al. 2009). Hence recombinant Elisa was superior to other serological assays because Elisa based on recombinant antigens are well known to offer higher reproducibility's, lack of cross-reactivity, easy to optimize, less labor intensive and do not require the cultivation of virus (MOHAN et al. 2006). In the present study**,** P1 and rHA1 polypeptides were used to study the possibility to use them in indirect rElisa for detection of antibodies towards H5. rHA1- Elisa proved to be highly sensitive and specific, as compared with HI, AGID and Western blot. Although Western blotting is sensitive (100 % in this study), blotting for large number of samples would be tedious, time consuming and precise quantification of antibody levels would not be feasible. In this study AGID test revealed to be less sensitive to detect H5 antibodies which in accordance with the results obtained by (MEULEMANS et al. 1987; SNYDER et al. 1985). Whole H5N1 antigen based Elisa showed high sensitivity to influenza A subtype H5 but it does not fulfil our purpose to produce safe antigen. Serial dilution Elisa was done to choose the best serum dilution for single dilution Elisa, hence, the use of single dilution Elisa reduces reagents, costs and time and the error inherent to serial dilution Elisa. It is worthy to mention that, negative sera was obtained from chickens with a history of vaccination against ND, IBD, IB and MD and there were no cross reactivity with rHA1- Elisa (antibodies towards ND and IBD were analyzed with HI and Elisa respectively. Elisa based on recombinant polypeptides, especially if these polypeptides are conserved in H5 might offers a considerable a advantages for detection of viral antibodies.The low sensitivity of P1 polypeptide (sensitivity 80 %) may be attributable to its small size (only 40 amino acid). However, the immunogenicity of such small polypeptides could be improved by cloning with different copies of insert which called tandem peptide (BEACHEY et al. 1987). The increase in the copy number of the coding sequence was important for antibody binding activity (WANG et al. 2010).

Elisa test was adopted as a sensitive method for the detection of antibodies to AIV and the results of Elisa test should be interpreted on a flock and not on individual bird bases**.** In case of H5N1 HPAI outbreaks, chickens shows clinical signs very severely and then die which mean that Elisa might has no significant value in eradication program. However, vaccination of chickens against HPAI must be considered to be a complement to other control measures, as part of a science-based influenza control

strategy (CAPUA and MARANGON 2003). rHA1-Elisa could be used for the surveillance immune responses by detecting antibodies to AIV. On the other hand, HPAI viruses do not show high virulence for all species of birds, and the clinical severity seen in any host appears to vary with both bird species and virus strain (ALEXANDER et al. 1978; ALEXANDER et al. 1986). In particular, ducks rarely show clinical signs as a result of HPAI infections. Ostriches (*Struthio camelus*) also appear to show mild clinical response to HPAI infection. rElisa significantly improves the ability for quickly detection of the antibody levels of AIV during field outbreaks and providing key information for disease control decision making, thus it has great application potential in long-term prevention and control of AIV as recommended**.** rHA1- Elisa shows high OD with positive duck sera (positive also by HI) as compared with OD of negative duck serum which indicate the validity of rHA1-Elisa to detect H5 antibodies in duck sera.

In conclusion; our findings recommend the use of the rHA1-Elisa as a tool for improvement of serological diagnosis of influenza A subtype H5 in chickens and ducks. But it remains a question of value whether rHA1-Elisa is specific for influenza A subtype H5? Additional studies are needed to further evaluate the rElisa in different avian species. The possibilities to use these recombinant polypeptides as a vaccine against H5 influenza should be further studied.

6 SUMMARY

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Truncated Sequences of Influenza Subtype H5 Haemagglutinin for Vaccination and Diagnostic Purposes

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Keywords: Avian influenza, Yeast expression, Peptide vaccination, recombinant Elisa

The highly pathogenic Avian Influenza subtype H5N1 can lead to 100 % mortality in chickens. The main issue in prevention of H5N1 is the development of efficient poultry vaccines. Influenza haemagglutinin (HA) derived recombinant polypeptides would not elicit an immune response against internal viral proteins. Thus HA polypeptide use facilitates differentiation between infected and vaccinated animals (DIVA). Serological tests using recombinant immune-dominant proteins devoid of non-specific moieties present in whole cell preparations might have higher sensitivity and specificity. In the present study, four non-overlapping sequences of different functional domains of influenza A virus subtype H5 virus (A / Thailand / 1 (Kan-1) / 2004) designated P1, P2, P5 and rHA1 were cloned and expressed in *Pichia pastoris* for vaccination and diagnosis purposes.

- The four polypeptides were expressed successfully in *P. pastoris* using peptone methanol (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (v/v) methanol). P1, P2 and rHA1 polypeptides were purified using nickel affinity chromatography, whereas, P5 was purified using lectin affinity chromatography. Correct expression was analysed by SDS-PAGE and western blot, glycosylation analysis and MALDI-TOF.
- The immune responses of P1, P2 and rHA1 polypeptides were assessed in BALB/C mice. To enhance antibody response, recombinant polypeptides were mixed with the Gerbu adjuvant and injected subcutaneously. Vaccination of mice induced high subtype specific antibody titres in mice as analysed by Elisa (using recombinant antigens or whole H5N1 antigen) and Immunofluorescence assay (IFA) performed on Vero cells infected with H5 (A / Thailand / 1 (Kan-1) / 2004).
- The immunogenicity of P1, P2, P5 and rHA1 polypeptides was determined in commercial layer chickens. Results showed that P1, P2 and rHA1 polypeptides

induced high subtype specific antibody titres in chickens as analysed by Elisa (using recombinant antigens or whole H5N1 antigen), IFA (performed on Vero cells infected with H5N1 A / Thailand / 1 (Kan-1) / 2004) and microneutralization test (µNT). However, P5 polypeptide was not immunogenic in chickens. Neutralizing antibodies could be detected in chicken sera immunized with P1, P2 and rHA1 polypeptides as analyzed with microneutralization test.

-IgY was analysed in egg yolk of chickens immunized with recombinant polypeptides. The IgY of chicken immunized with P1 and rHA1, transferred to the egg yolk was proportional to maternal serum IgY. However, IgY could not be detected in egg yolk of chickens immunized with P2 and P5 recombinant polypeptides

- The more immunogenic polypeptides P1 and rHA1 were used in an recombinant Elisa (rElisa) for detection of influenza A subtype H5 in chickens and duck sera.The optimal antigen for the concentrations of rHA1, P1 was 50 ng / well, 50 ng / well.

- Analysis of 25 positive sera and 25 negative sera to H5 antibodies revealed that, the sensitivity of Western blot, whole H5N1 Elisa, agar gel immunodiffusion test (AGID), P1-Elisa and rHA1-Elisa was 100 %, 100 %, 52 %, 80 % and 100 %, respectively, while the specificity was 100 %, 100 %, 100 %, 72 %, and 100 %, respectively. Moreover, duck sera, with haemagglutination inhibiting titer ranged from 4 - 8 $\log_2$, were tested positive by rHA1 Elisa compared with negative duck sera.

-Further analysis of 179 serum samples with rHA1-Elisa in comparison with haemagglutination inhibition (HI) and commercial Elisa proved to be highly sensitive and specific. The agreement ratio between rElisa and HI was 84.9 % and between commercial Elisa (Flock check) and HI was 76.5 %.

In conclusion, *P. pastoris* may allow development of an effective recombinant influenza vaccine based on truncated sequences of HA that might provide broader protection against H5 influenza viruses. The possibilities to use rHA1, P1 and P5 recombinant polypeptides as a vaccine against H5 influenza should be further studied. Also our study demonstrates the potential utility of recombinant Elisa as a tool for improvement of serological diagnosis of influenza A subtype H5 in chickens and ducks.

7 Zusammenfassung

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Peptide des Hämagglutinin- Proteins von Influenza A Virus Subtyp H5 für Impfstoff- und Diagnosezwecke

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Schlüsselwörter:

Vogelgrippe, Hefe-Expression, Peptid-Vakzination, rekombinante ELISA

Die hochpathogene aviäre Influenza des Subtyps H5N1 erreicht beim Ausbruch von Infektionen in Nutzgeflügelbeständen Mortalitätsraten von bis zu 100 %. Effektive und kostengünstige Impfstoffe werden benötigt, die möglichst auch eine Differenzierung zwischen geimpften Tieren und mit Wild-Virus infizierten Tieren zulassen. In diesem Zusammenhang könnten Peptid-Vakzine eine mögliche Alternative zu den herkömmlichen Impfstoffen darstellen, bei denen unter Verwendung des Vollvirus Antikörper gegen mehrere Virusproteine induziert werden. Außerdem, könnten rekombinante Antigene in serologischen Tests zur Diagnose von H5 Virus in Nutzgeflügel eingesetzt werden. Von dem Einsatz spezifischer rekombinanter Antigene ist eine Verbesserung der Serodiagnostik zu erwarten. In dieser Arbeit, wurden vier verkürzte Sequenzen des Hämagglutinins (P1, P2, P5 und rHA1) von Subtyp H5 (A / Thailand / 1 (Kan-1) / 2004) rekombinant in *Pichia Pastoris* exprimiert.

- Dazu erfolgten zunächst eine Klonierung in der Expressionsvektor pAOX und die Transformation von *Pichia Pastoris*. Die Expression wurde durch Methanol induziert. Der Nachweis der rekombinanten Fusionspeptiden mit C-terminalen Histidin-Tag erfolgte durch SDS-PAGE, Western Blot, Glycolysierungsanalyse, und MALDI-TOF. Der Histidin-Tag ermöglichte die Reinigung von P1, P2 und rHA1 mit Metall-Affinitätschromatographie. Polypeptid P5 hingegen wurde mittels Lectin-Affinitäts-chromatographie gereinigt.
- Balb/c Mäuse wurden mit Polypeptid P1, P2 bzw. rHA1, versetzt mit Gerbu Adjuvans, immunisiert. Zur Untersuchung der Immunantwort wurden die murinen Seren mittels Elisa (unter Verwendung rekombinanter Antigene oder Voll-H5N1 Antigen) sowie IFA

(durchgeführt in Vero- Zellen infiziert mit A / Thailand / 1 (Kan-1) / 2004) analysiert. Dabei wurde die präferentielle Induktion von H5-spezifischen Antikörpern detektiert.

- Die Immunogenität der P1, P2, P5 und rHA1-Polypeptide wurde in kommerziellen Legehennen bestimmt. Seren wurden mit ELISA, IFA, und Mikroneutralizationstest (µNT) analysiert. Die ELISA-Ergebnisse zeigten, dass die Polypeptide P1, P2 und rHA1 hohe Subtyp-spezifische Antikörpertiter in Hühnern induzierten. Im µNT konnte nur ein niedriger neutralisierender Antikörpertiter nachgewiesen werden. Das P5-Polypeptid ist bei Hühnern nicht immunogen.
- Im Eigelb von Hühnern, die mit den rekombinanten Polypeptiden P1 und rHA1 immunisiert wurden, konnten H5-spezifische IgY Antikörper detektiert werden. Hühner, die mit P2 und P5 immunisiert wurden, zeigten keine IgY im Eigelb.
- Die rekombinanten Antigene P1 und rHA1 wurden im ELISA auf ihre potenzielle Eignung für die Serodiagnostik untersucht. Die optimale Antigenkonzentration war 50 ng / well. Die serologische Analyse von 25 positiven und 25 negativen Seren auf Antikörper gegen H5 zeigte, dass Sensitivität und Spezifität von Western Blot, Voll-H5N1 ELISA und rHA1-ELISA bei jeweils 100 % lagen. Bei Agargel-Immunodiffusiontest (AGID) lagen Sensitivität und Spezifität bei 52 % und 100 %, während im P1-Elisa lediglich eine Sensitivität von 80 % und eine Spezifität von 72 % erreicht wurden. Somit eignet sich rHA1 für die Anwendung in der Serodiagnostik.
- Bei der serologischen Untersuchung von 175 Hühnerseren wurde eine Überbestimmung zwischen rHA1-ELISA und Hämagglutinationshemmungstest (HAI) 84.9 % festgestellt, während diese zwischen dem kommerziellen ELISA (Flock Check) und HAI 76.5 % betrug.
- Die Ergebnisse zeigten, dass das Expressionssystem *P. pastoris* als Produktionssystem rekombinanter Antigene für die Serodiagnostik von H5 Influenza geeignet ist. Challenge-Versuche sind nötig, um die Eignung von rekombinanten Antigenen als möglichen Impfstoff gegen H5 Influenza zu untersuchen.

REFERENCES

Abraham A, Sivanandan V , Karunakaran D , Halvorson DA , Newman J A. Comparative serological evaluation of avian influenza vaccine in turkeys. Avian Dis. 1988; 32 :659-62.

Acland HM, Silverman Bachin LA, Eckroade RJ. Lesions in broiler and layer chickens in an outbreak of highly pathogenic avian influenza virus infection. Vet Pathol. 1984; 21 :564-9.

Adair BM, Todd D, McKillop ER, McNulty MS. Detection of influenza a type-specific antibodies in chicken and turkey sera by enzyme linked immunosorbent assay. Avian Pathol. 1989; 18 :455-63.

Al-Natour MQ, Ward LA, Saif YM, Stewart-Brown B, Keck LD. Effect of diffferent levels of maternally derived antibodies on protection against infectious bursal disease virus. Avian Dis. 2004; 48 :177-182.

Alexander DJ. A review of avian influenza in different bird species. Vet Microbiol. 2000; 74 :3-13.

Alexander DJ. Avian influenza viruses and human health. Dev Biol (Basel). 2006; 124 :77-84.

Alexander DJ. An overview of the epidemiology of avian influenza. Vaccine. 2007; 25 :5637-44.

Alexander DJ, Allan WH, Parsons DG, Parsons G. The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. Res Vet Sci. 1978; 24 :242-7.

Alexander DJ, Parsons G, Manvell RJ. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. Avian Pathol. 1986; 15 :647-62.

Ali A, Avalos RT, Ponimaskin E, Nayak DP. Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. J Virol. 2000; 74 :8709-19.

Allan WH, Madeley CR, Kendal AP. Studies with avian influenza A viruses: cross protection experiments in chickens. J Gen Virol. 1971; 12 :79-84.

Altstein AD, Gitelman AK, Smirnov YA, Piskareva LM, Zakharova LG, Pashvykina GV, Shmarov MM, Zhirnov OP, Varich NP, Ilyinskii PO, Shneider AM. Immunization with influenza A NP-expressing vaccinia virus recombinant protects mice against experimental infection with human and avian influenza viruses. Arch Virol. 2006; 151 :921-31.

Avellaneda G, Mundt E, Lee CW, Jadhao S, Suarez DL. Differentiation of infected and vaccinated animals (DIVA) using the NS1 protein of avian influenza virus. Avian Dis. 2010; 54 :278-86.

Aymard M, Ferraris O, Gerentes L, Jolly J, Kessler N. Neuraminidase assays. Dev Biol (Basel). 2003; 115 :75-83.

Babiuk S, Albrecht R, Berhane Y, Marszal P, Richt JA, Garcia-Sastre A, Pasick J, Weingartl H. 1918 and 2009 H1N1 influenza viruses are not pathogenic in birds. J Gen Virol. 2010; 91 :339-42.

Bahgat MM, Kutkat MA, Nasraa MH, Mostafa A, Webby R, Bahgat IM, Ali MA. Characterization of an avian influenza virus H5N1 Egyptian isolate. J Virol Methods. 2009; 159 :244-50.

Baigent SJ, McCauley JW. Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. Bioessays. 2003; 25 :657-71.

Bano S, Naeem K, Malik SA. Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. Avian Dis. 2003; 47 :817-22.

Barr DA, Kelly AP, Badman RT, Campey AR, O'Rourke MD, Grix DC, Reece RL. Avian influenza on a multi-age chicken farm. Aust Vet J. 1986; 63 :195-6.

Barry MA, Johnston SA. Biological features of genetic immunization. Vaccine. 1997; 15 :788-91.

Bauer CM, Pinto LH, Cross TA, Lamb RA. The influenza virus M2 ion channel protein: probing the structure of the transmembrane domain in intact cells by using engineered disulfide cross-linking. Virology. 1999; 254 :196-209.

Beachey EH, Seyer JM, Dale JB. Protective immunogenicity and T lymphocyte specificity of a trivalent hybrid peptide containing NH2-terminal sequences of types 5, 6, and 24 M proteins synthesized in tandem. J Exp Med. 1987; 166 :647-56.

Bean WJ, Kawaoka Y, Wood JM. Pearson JE, Webster RG. Characterization of virulent and avirulent A/chicken/Pennsylvania/83 influenza A viruses: potential role of defective interfering RNAs in nature. J Virol. 1985; 54 :151-60.

Beard, C.V. Isolation and identification of avian pathogens. In: Hitchner, S.B., Domermuth, C.H., Purchase, H.G. and Williams, J.E. (ed.). 1998; 67-9.

Beck JR, Swayne DE, Davison S, Casavant S, Gutierrez C. Validation of egg yolk antibody testing as a method to determine influenza status in white leghorn hens. Avian Dis. 2003; 47 :1196-9.

Birch-Machin I, Rowan A, Pick J, Mumford J, Binns M. Expression of the nonstructural protein NS1 of equine influenza A virus: detection of anti-NS1 antibody in post infection equine sera. J Virol Methods. 1997; 65 :255-63.

Bot A, Bot S, Garcia-Sastre A, Bona C. DNA immunization of newborn mice with a plasmid-expressing nucleoprotein of influenza virus. Viral Immunol. 1996; 9 :207-210.

Bouvier NM, Palese P. The biology of influenza viruses. Vaccine. 2008; 4 :49-53.

Boyle DB, Selleck P, Heine HG. Vaccinating chickens against avian influenza with fowlpox recombinants expressing the H7 haemagglutinin. Aust Vet. 2000; 78 :44-8.

Brake AJ, Julius DJ, Thorner J. A functional prepro-alpha-factor gene in Saccharomyces yeasts can contain three, four, or five repeats of the mature pheromone sequence. Mol Cell Biol. 1983; 3 :1440-50.

Broekhuijsen MP, van Rijn JM, Blom AJ, Pouwels PH, Enger-Valk BE, Brown F, Francis MJ. Fusion proteins with multiple copies of the major antigenic determinant of foot-and-mouth disease virus protect both the natural host and laboratory animals. J Gen Virol. 1987; 68 :3137-43.

Brown DW, Kawaoka Y, Webster RG, Robinson HL. Assessment of retrovirus-expressed nucleoprotein as a vaccine against lethal influenza virus infections of chickens. Avian Dis. 1992; 36 :515-20.

Brown EG. Influenza virus genetics. Biomed Pharmacother. 2000; 54, 196-209.

Brugh M, Beard CW, Stone HD. Immunization of chickens and turkeys against avian influenza with monovalent and polyvalent oil emulsion vaccines. Am J Vet Res. 1979; 40 :165-9.

Bublot M, Pritchard N, Cruz JS, Mickle TR, Selleck P, Swayne DE. Efficacy of a fowlpox-vectored avian influenza H5 vaccine against Asian H5N1 highly pathogenic avian influenza virus challenge. Avian Dis. 2007; 51 :498-500.

Busquets N, Abad FX, Alba A, Dolz R, Allepuz A, Rivas R, Ramis A, Darji A, Majo N. Persistence of highly pathogenic avian influenza virus (H7N1) in infected chicken: feather as a suitable sample for diagnosis. J Gen Virol. 2010. in Press.

Capua I, Alexander DJ. The challenge of avian influenza to the veterinary community. Avian Pathol. 2006; 35 :189-205.

Capua I, Alexander DJ. Animal and human health implications of avian influenza infections. Biosci Rep. 2007; 27 :359-72.

Capua I, Alexander DJ. Avian influenza infection in birds: a challenge and opportunity for the poultry veterinarian. Poult Sci. 2009; 88 :842-6.

Capua I, Marangon S. Vaccination policy applied for the control of avian influenza in Italy. Dev Biol (Basel). 2003; 114 :213-9.
Capua I, Marangon S. The challenge of controlling notifiable avian influenza by means of vaccination. Avian Dis. 2007a, 51 :317-22.
Capua I, Marangon S. Control and prevention of avian influenza in an evolving scenario. Vaccine. 2007b; 25 :5645-52.
Capua I, Marangon S, Dalla Pozza M, Santucci U. Vaccination for avian influenza in Italy. Vet Rec. 2000; 147 :751.
Capua I, Mutinelli F. Mortality in Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian Pathol. 2001; 30 :179-83.
Capua I, Schmitz A, Jestin V, Koch G, Marangon S. Vaccination as a tool to combat introductions of notifiable avian influenza viruses in Europe, 2000 to 2006. Rev Sci Tech. 2009; 28 :245-59.
Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez JF. Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol. 2003; 32 :47-55.
Cardona CJ, Charlton BR, Woolcock PR. Persistence of immunity in commercial egg-laying hens following vaccination with a killed H6N2 avian influenza vaccine. Avian Dis. 2006; 50 :374-9.
Cassetti MC, Noah DL, Montelione GT, Krug RM. Efficient translation of mRNAs in influenza A virus-infected cells is independent of the viral 5' untranslated region. Virology. 2001; 289 :180-5.
Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell. 1982; 31 : 17-27.
Cattoli G, Milani A, Bettini F, Serena Beato M, Mancin M, Terregino C, Capua I. Development and validation of an anti-N3 indirect immunofluorescent antibody test to be used as a companion diagnostic test in the framework of a "DIVA" vaccination strategy for avian influenza infections in poultry. Avian Pathol. 2006; 35 :154-9.
Cattoli G, Drago A, Maniero S, Toffan A, Bertoli E, Fassina S, Terregino C, Robbi C, Vicenzoni G, Capua I. Comparison of three rapid detection systems for type A influenza virus on tracheal swabs of experimentally and naturally infected birds. Avian Pathol. 2004; 33 :432-7.

Cauthen AN, Swayne DE, Schultz-Cherry S, Perdue ML, Suarez DL. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J Virol. 2000; 74 :6592-9.

Cereghino GP, Cereghino JL, Ilgen C, Cregg JM. Production of recombinant proteins in fermenter cultures of the yeast Pichia pastoris. Curr Opin Biotechnol. 2002; 13 :329-32.

Chambers TM, Kawaoka Y, Webster RG. Protection of chickens from lethal influenza infection by vaccinia-expressed hemagglutinin. Virology. 1988; 167 :414-421.

Chan SK, Rees DC. Molecular basis for the alpha1-protease inhibitor deficiency. Nature. 1975; 255 :240-1.

Chen J, Deng YM. Influenza virus antigenic variation, host antibody production and new approach to control epidemics. Virol J. 2009; 6 :30.

Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell. 1998a; 95 :409-17.

Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med. 2001; 7 :1306-12.

Chen Z. 2004, Influenza DNA vaccine: an update. Chin Med J (Engl). 2004; 117 :125-32.

Chen Z, Sahashi Y, Matsuo K, Asanuma H, Takahashi H, Iwasaki T, Suzuki Y, Aizawa C, Kurata T, Tamura S. Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza. Vaccine. 1998b; 16 :1544-9.

Cherbonnel M, Rousset J, Jestin V. Strategies to improve protection against low-pathogenicity H7 avian influenza virus infection using DNA vaccines. Avian Dis. 2003; 47 :1181-6.

Chiu FF, Venkatesan N, Wu CR, Chou AH, Chen HW, Lian SP, Liu SJ, Huang CC, Lian WC, Chong P, Leng CH. Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus. Biochem Biophys Res Commun. 2009; 383 :27-31.

Claas EC, de Jong JC, van Beek R, Rimmelzwaan GF, Osterhaus AD. Human influenza virus A/HongKong/156/97 (H5N1) infection. Vaccine. 1998; 16 :977-8.

Collins RA, Ko LS, So KL, Ellis T, Lau LT, Yu AC. Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA. J Virol Methods. 2002; 103 :213-25.

Cox MM. Cell-based protein vaccines for influenza. Curr Opin Mol Ther. 2005; 7 :24-9.

Crawford JM, Garcia M, Stone H, Swayne D, Slemons R, Perdue ML. Molecular characterization of the hemagglutinin gene and oral immunization with a waterfowl-origin avian influenza virus. Avian Dis. 1998; 42 :486-96.

Cristalli A, Capua I. Practical problems in controlling H5N1 high pathogenicity avian influenza at village level in Vietnam and introduction of biosecurity measures. Avian Dis. 2007; 51 :461-2.

Davison S, Ziegler AF, Eckroade RJ. Comparison of an antigen-capture enzyme immunoassay with virus isolation for avian influenza from field samples. Avian Dis. 1998; 42 :791-5.

Daly R, Hearn MT. Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production. J Mol Recognit. 2005; 18 :119-38.

De BK, Shaw MW, Rota PA, Harmon MW, Esposito JJ, Rott R, Cox NJ, Kendal AP. Protection against virulent H5 avian influenza virus infection in chickens by an inactivated vaccine produced with recombinant vaccinia virus. Vaccine. 1988; 6 :257-61.

de Jong MD. Hien TT. Avian influenza A (H5N1). J Clin Virol. 2006; 35 :2-13.

Deshpande KL, Fried VA, Ando M, Webster RG. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. Proc Natl Acad Sci U S A. 1987; 84 :36-40.

Di Trani L, Cordioli P, Falcone E, Lombardi G, Moreno A, Sala G, Tollis M. Standardization of an inactivated H17N1 avian influenza vaccine and efficacy against A/Chicken/Italy/13474/99 high-pathogenicity virus infection. Avian Dis. 2003; 47 :1042-6.

DiMarchi R, Brooke G, Gale C, Cracknell V, Doel T, Mowat N. Protection of cattle against foot-and-mouth disease by a synthetic peptide. Science. 1986; 232 :639-41.

DiNapoli JM, Nayak B, Yang L, Finneyfrock BW, Cook A, Andersen H, Torres-Velez F, Murphy BR, Samal SK, Collins PL, Bukreyev A. Newcastle disease virus-vectored vaccines expressing the hemagglutinin or neuraminidase protein of H5N1 highly

pathogenic avian influenza virus protect against virus challenge in monkeys. J Virol. 2010; 84 :1489-503.

DiNapoli JM, Yang L, Suguitan A, Elankumaran S, Dorward DW, Murphy BR, Samal SK, Collins PL, Bukreyev A. Immunization of primates with a Newcastle disease virus-vectored vaccine via the respiratory tract induces a high titer of serum neutralizing antibodies against highly pathogenic avian influenza virus. J Virol. 2007; 81 :11560-8.

Ding, J, Bao W, Liu Q, Yu Q, Abdille MH, Wei Z. Immunoprotection of chickens against Eimeria acervulina by recombinant alpha-tubulin protein. Parasitol Res. 2008; 103 :1133-40.

Dlugolenski D, Hauck R, Hogan RJ, Michel F, Mundt E. Production of H5-specific monoclonal antibodies and the development of a competitive enzyme-linked immunosorbent assay for detection of H5 antibodies in multiple species. Avian Dis. 2010; 54 :644-9.

Dybkaer K, Munch M, Handberg KJ, Jorgensen PH. Application and evaluation of RT-PCR-ELISA for the nucleoprotein and RT-PCR for detection of low-pathogenic H5 and H7 subtypes of avian influenza virus. J Vet Diagn Invest. 2004; 16 :51-6.

Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. Science. 2009; 324 :246-51.

Elbers AR, Koch G, Bouma A. Performance of clinical signs in poultry for the detection of outbreaks during the avian influenza A (H7N7) epidemic in The Netherlands in 2003. Avian Pathol. 2005; 34 :181-7.

Ellis TM, Leung CY, Chow MK, Bissett LA, Wong W, Guan Y, Malik Peiris JS. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol. 2004; 33 :405-12.

Enami K, Qiao Y, Fukuda R, Enami M. An influenza virus temperature-sensitive mutant defective in the nuclear-cytoplasmic transport of the negative-sense viral RNAs. Virology. 1993; 194 :822-7.

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

Erbel PJ, Karimi-Nejad Y, De Beer T, Boelens R, Kamerling JP, Vliegenthart JF. Solution structure of the alpha-subunit of human chorionic gonadotropin. Eur J Biochem. 1999; 260 :490-8.

Erbel PJ, Karimi-Nejad Y, van Kuik JA, Boelens R, Kamerling JP, Vliegenthart JF. Effects of the N-linked glycans on the 3D structure of the free alpha-subunit of human chorionic gonadotropin. Biochemistry. 2000; 39 :6012-21.

Errington W, Steward M, Emmerson PT. A diagnostic immunoassay for Newcastle disease virus based on the nucleocapsid protein expressed by a recombinant baculovirus. J Virol Methods. 1995; 55 :357-65.

Eshaghi S, Hedren M, Nasser MI, Hammarberg T, Thornell A, Nordlund P. An efficient strategy for high-throughput expression screening of recombinant integral membrane proteins. Protein Sci. 2005; 14 :676-83.

Fatunmbi OO, Newman JA, Sivanandan V, Halvorson DA. Enhancement of antibody response of turkeys to trivalent avian influenza vaccine by positively charged liposomal avridine adjuvant. Vaccine. 1992; 10:623-6.

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 2005; 79 :2814-22.

Fouchier RA, Bestebroer TM, Herfst S, Van Der Kemp L, Rimmelzwaan GF, Osterhaus AD. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. J Clin Microbiol. 2000; 38 :4096-101.

Fynan EF, Robinson HL, Webster RG. Use of DNA encoding influenza hemagglutinin as an avian influenza vaccine. DNA Cell Biol. 1993; 12 :785-9.

Gao W, Soloff AC, Lu X, Montecalvo A, Nguyen DC, Matsuoka Y, Robbins PD, Swayne DE, Donis RO, Katz JM, Barratt-Boyes SM, Gambotto A. Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. J Virol. 2006; 80 :1959-64.

Gares SL, Fischer KP, Congly SE, Lacoste S, Addison WR, Tyrrell DL, Gutfreund KS. Immunotargeting with CD154 (CD40 ligand) enhances DNA vaccine responses in ducks. Clin Vaccine Immunol. 2006; 13 :958-65.

Ge J, Deng G, Wen Z, Tian G, Wang Y, Shi J, Wang X, Li Y, Hu S, Jiang Y, Yang C, Yu, K, Bu Z, Chen H. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. J Virol. 2007; 81 :150-8.

Gomez-Puertas P, Albo C, Perez-Pastrana E, Vivo A, Portela A. Influenza virus matrix protein is the major driving force in virus budding. J Virol. 2000; 74 :11538-47.
Gregoriades A, Frangione B. Insertion of influenza M protein into the viral lipid bilayer and localization of site of insertion. J Virol. 1981; 40 :323-8.
Grodecka M, Czerwinski M, Duk M, Lisowska E, Wasniowska K. Analysis of recombinant Duffy protein-linked N-glycans using lectins and glycosidases. Acta Biochim Pol. 2010; 57 :49-53.
Gunther I, Glatthaar B, Doller G, Garten W. A H1 hemagglutinin of a human influenza A virus with a carbohydrate-modulated receptor binding site and an unusual cleavage site. Virus Res. 1993; 27 :147-60.
Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization. Annu Rev Immunol. 2000, 18 :927-74.
Hampson AW, Mackenzie JS. The influenza viruses. Med J Aust. 2006; 185, S39-43.
Harris A, Forouhar F, Qiu S, Sha B, Luo M. The crystal structure of the influenza matrix protein M1 at neutral pH: M1-M1 protein interfaces can rotate in the oligomeric structures of M1. Virology. 2001; 289 :34-44.
Hay AJ, Kennedy NC, Skehel JJ, Appleyard G. The matrix protein gene determines amantadine-sensitivity of influenza viruses. J Gen Virol. 1979; 42 :189-91.
Hghihghi HR, Read LR, Mohammadi H, Pei Y, Ursprung C, Nagy E, Behboudi S, Haeryfar SM, Sharif S. Characterization of host responses against a recombinant fowlpox virus-vectored vaccine expressing the hemagglutinin antigen of an avian influenza virus. Clin Vaccine Immunol. 2010; 17:454-63.
Hioe CE, Hinshaw VS. Induction and activity of class II-restricted, Lyt-2+ cytolytic T lymphocytes specific for the influenza H5 hemagglutinin. J Immunol. 1989; 142 :2482-8.
Hitzeman RA, Chen CY, Dowbenko DJ, Renz ME, Liu C, Pai R, Simpson NJ, Kohr WJ, Singh A, Chisholm V et al. Use of heterologous and homologous signal sequences for secretion of heterologous proteins from yeast. Methods Enzymol. 1990; 185 :421-40.
Hitzeman RA, Hagie FE, Levine HL, Goeddel DV, Ammerer G, Hall BD. Expression of a human gene for interferon in yeast. Nature. 1981; 293 :717-22.
Hoelscher MA, Garg S, Bangari DS, Belser JA, Lu X, Stephenson I, Bright RA, Katz JM, Mittal SK, Sambhara S. Development of adenoviral-vector-based pandemic

influenza vaccine against antigenically distinct human H5N1 strains in mice. Lancet. 2006; 367 :475-81.
Hoffmann E, Lipatov AS, Webby RJ, Govorkova EA, Webster RG. Role of specific hemagglutinin amino acids in the immunogenicity and protection of H5N1 influenza virus vaccines. Proc Natl Acad Sci U S A. 2005; 102 :12915-20.
Holsinger LJ, Lamb RA. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. Virology. 1991; 183 :32-43.
Homme PJ, Easterday BC. Avian influenza virus infections. IV. Response of pheasants, ducks, and geese to influenza A-turkey-Wisconsin-1966 virus. Avian Dis. 1970; 14 :285-90.
Hooper PT. Lesions in chickens experimentally infected with 1985 H7N7 avian influenza virus. Aust Vet J. 1989; 66 :155-6.
Hooper PT, Russell GW, Selleck PW, Stanislawek WL. Observations on the relationship in chickens between the virulence of some avian influenza viruses and their pathogenicity for various organs. Avian Dis. 1995; 39 :458-64.
Horie M, Ogawa H, Yamada K, Hara A, Bui VN, Awad SS, Yoshikawa R, Mase M, Tsukamoto K, Yamaguchi S, Nakamura K, Imai K. A latex agglutination test using a recombinant nucleoprotein for detection of antibodies against avian influenza virus. J Virol Methods. 2009; 161 :259-64.
Horimoto T, Rivera E, Pearson J, Senne D, Krauss S, Kawaoka Y, Webster RG. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology. 1995; 213 :223-30.
Horvath A, Toth GK, Gogolak P, Nagy Z, Kurucz I, Pecht I, Rajnavolgyi E. A hemagglutinin-based multipeptide construct elicits enhanced protective immune response in mice against influenza A virus infection. Immunol Lett. 1998; 60 :127-36.
Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, Tang J, McDonald W, Song L, Evans RK, Umlauf S, Tussey L, Powell TJ. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine. 2008; 26 :201-14.
Hunt LA, Brown DW, Robinson HL, Naeve CW, Webster RG. Retrovirus-expressed hemagglutinin protects against lethal influenza virus infections. J Virol. 1988; 62 :3014-9.

Jackwood MW, Suarez DL, Hilt D, Pantin-Jackwood MJ, Spackman E, Woolcock P Cardona C. Biologic characterization of chicken-derived H6N2 low pathogenic avian influenza viruses in chickens and ducks. Avian Dis. 2010; 54 :120-5.

Jayawardane GW, Spradbrow PB. Mucosal immunity in chickens vaccinated with the V4 strain of Newcastle disease virus. Vet Microbiol. 1995; 46 :69-77.

Jeong OM, Kim MC, Kang HM, Ha GW, Oh JS, Yoo JE, Park CH, Kwon JS, Pack MR, Kim HR, Kim YJ, Kwon JH, Lee YJ. Validation of egg yolk antibody based C-ELISA for avian influenza surveillance in breeder duck. Vet Microbiol. 2010.

Jin M, Wang G, Zhang R, Zhao S, Li H, Tan Y, Chen H. Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. Avian Dis. 2004; 48 :870-8.

Johansson BE. Immunization with influenza A virus hemagglutinin and neuraminidase produced in recombinant baculovirus results in a balanced and broadened immune response superior to conventional vaccine. Vaccine. 1999; 17: 2073-80.

Joseph T, McAuliffe J, Lu B, Vogel L, Swayne D, Jin H, Kemble G, Subbarao K. A live attenuated cold-adapted influenza A H7N3 virus vaccine provides protection against homologous and heterologous H7 viruses in mice and ferrets. Virology. 2008; 378 :123-32.

Karunakaran D, Newman JA, Halvorson DA, Abraham A. Evaluation of inactivated influenza vaccines in market turkeys. Avian Dis. 1987; 31:498-503.

Kashyap AK, Steel J, Oner AF, Dillon MA, Swale RE, Wall KM, Perry KJ, Faynboym A, Ilhan M, Horowitz M, Horowitz L, Palese P, Bhatt RR, Lerner RA. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc Natl Acad Sci U S A. 2008; 105 :5986-91.

Kaspers B, Schranner I, Losch U. Distribution of immunoglobulins during embryogenesis in the chicken. Zentralbl Veterinarmed A. 1991; 38 :73-9.

Katz JM, Lu X, Frace AM, Morken T, Zaki SR, Tumpey TM. Pathogenesis of and immunity to avian influenza A H5 viruses. Biomed Pharmacother. 2000; 54 :178-87.

Kaverin NV, Rudneva IA, Ilyushina NA, Varich NL, Lipatov AS, Smirnov YA, Govorkova EA, Gitelman AK, Lvov DK, Webster RG. Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. J Gen Virol. 2002; 83 :2497-505.

Kawaoka Y, Webster RG. Molecular mechanism of acquisition of virulence in influenza virus in nature. Microb Pathog. 1988; 5 :311-8.
Kida H, Brown LE, Webster RG. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology. 1982; 122 :38-47.
Kida H, Yanagawa R, Matsuoka Y. Duck influenza lacking evidence of disease signs and immune response. Infect Immun. 1980; 30 :547-53.
Kida H, Yoden S, Kuwabara M, Yanagawa R. Interference with a conformational change in the haemagglutinin molecule of influenza virus by antibodies as a possible neutralization mechanism. Vaccine. 1985; 3 :219-22.
Kido H, Yokogoshi Y, Sakai K, Tashiro M, Kishino Y, Fukutomi A, Katunuma N. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J Biol Chem. 1992; 267 :3573-9.
Kim JK, Negovetich NJ, Forrest HL, Webster RG. Ducks: the "Trojan horses" of H5N1 influenza. Influenza Other Respi Viruses. 2009; 3 :121-8.
Kim JN, Byun SH, Kang SY, Mo IP. Evaluation of neuraminidase antigen based competitive enzyme-linked immunosorbent assay in chickens vaccinated with avian influenza inactivated vaccine. Avian Dis. 2010; 54 :682-5.
Kim SH, Samal S. Inhibition of Host Innate Immune Responses and Pathogenicity of Recombinant Newcastle Disease Viruses Expressing NS1 Genes of Influenza A Viruses. J Gen Virol. 2010.
Kobayashi Y, Horimoto T, Kawaoka Y, Alexander DJ, Itakura C. Pathological studies of chickens experimentally infected with two highly pathogenic avian influenza viruses. Avian Pathol. 1996; 25 :285-94.
Kodihalli S, Haynes JR, Robinson HL, Webster RG. Cross-protection among lethal H5N2 influenza viruses induced by DNA vaccine to the hemagglutinin. J Virol. 1997; 71 : 3391-6.
Kodihalli S, Kobasa DL, Webster RG. Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines. Vaccine. 2000; 18 :2592-9.
Kowalczyk K, Daiss J, Halpern J, Roth TF. Quantitation of maternal-fetal IgG transport in the chicken. Immunology. 1985; 54 :755-62.
Kramer TT, Cho HC. Transfer of immunoglobulins and antibodies in the hen's egg. Immunology. 1970; 19 :157-67.

Kreijtz JH, Suezer Y, van Amerongen G, de Mutsert G, Schnierle BS, Wood JM, Kuiken T, Fouchier RA, Lower J, Osterhaus AD, Sutter G, Rimmelzwaan GF. Recombinant modified vaccinia virus Ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1. J Infect Dis. 2007; 195 :1598-606.

Kurjan J, Herskowitz I. Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. Cell. 1982; 30 :933-43.

Kwon YK, Joh SJ, Kim MC, Sung HW, Lee YJ, Choi JG, Lee EK, Kim JH, Highly pathogenic avian influenza (H5N1) in the commercial domestic ducks of South Korea. Avian Pathol. 2005; 34 :367-70.

Kyriakis CS, De Vleeschauwer A, Barbe F, Bublot M, Van Reeth K. Safety, immunogenicity and efficacy of poxvirus-based vector vaccines expressing the haemagglutinin gene of a highly pathogenic H5N1 avian influenza virus in pigs. Vaccine. 2009; 27:2258-64.

Lahariya C, Sharma AK, Pradhan SK. Avian flu and possible human pandemic. Indian Pediatr. 2006; 43 :317-25.

Lamb RA, Choppin PW. The gene structure and replication of influenza virus. Annu Rev Biochem. 1983; 52 :467-76.

Lamb RA, Takeda M. Death by influenza virus protein. Nat Med. 2001, 7, 1286-1288.

Latham T, Galarza JM. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. J Virol. 2001; 75 :6154-5.

Laudert E, Halvorson D, Sivanandan V, Shaw D. Comparative evaluation of tissue trophism characteristics in turkeys and mallard ducks after intravenous inoculation of type A influenza viruses. Avian Dis. 1993; 37 :773-80.

Laver WG, Webster RG. Preparation and immunogenicity of an influenza virus hemagglutinin and neuraminidase subunit vaccine. Virology. 1976; 69 :511-22.

Lee CW, Senne DA, Suarez DL. Development of hemagglutinin subtype-specific reference antisera by DNA vaccination of chickens. Avian Dis. 2003; 47:1051-6.

Lee CW, Senne DA, Suarez DL. Development and application of reference antisera against 15 hemagglutinin subtypes of influenza virus by DNA vaccination of chickens. Clin Vaccine Immunol. 2006; 13 :395-402.

Lee JW, Huang JP, Hong LS, Shu SF, Yu C, Chu CY. Prokaryotic recombinant hemagglutinin-neuraminidase protein enhances the humoral response and efficacy of commercial Newcastle disease vaccines in chickens. Avian Dis. 2010; 54 :53-8.
Lewis DB. Avian flu to human influenza. Annu Rev Med. 2006; 57 :139-44.
Ligon BL. Avian influenza virus H5N1: a review of its history and information regarding its potential to cause the next pandemic. Semin Pediatr Infect Dis. 2005; 16 : 326-35.
Lillehoj HS, Choi KD, Jenkins MC, Vakharia VN, Song KD, Han JY, Lillehoj EP. A recombinant Eimeria protein inducing interferon-gamma production: comparison of different gene expression systems and immunization strategies for vaccination against coccidiosis. Avian Dis. 2000; 44 :379-89.
Lin YJ, Deng MC, Wu SH, Chen YL, Cheng HC, Chang CY, Lee MS, Chien MS, Huang CC. Baculovirus-derived hemagglutinin vaccine protects chickens from lethal homologous virus H5N1 challenge. J Vet Med Sci. 2008; 70 :1147-52.
Lipatov AS, Andreansky S, Webby RJ, Hulse DJ, Rehg JE, Krauss S, Perez DR, Doherty PC, Webster RG, Sangster MY. Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses. J Gen Virol. 2005; 86 :1121-30.
Liu C, Eichelberger MC, Compans RW, Air GM. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol. 1995; 69 :1099-106.
Liu M, Wood JM, Ellis T, Krauss S, Seiler P, Johnson C, Hoffmann E, Humberd J, Hulse D, Zhang Y, Webster RG, Perez DR. Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. Virology. 2003; 314 :580-90.
Liu Y, Mundt E, Mundt A, Sylte M, Suarez DL, Swayne DE, Garcia M. Development and evaluation of an avian influenza, neuraminidase subtype 1, indirect enzyme-linked immunosorbent assay for poultry using the differentiation of infected from vaccinated animals control strategy. Avian Dis. 2010; 54 :613-21.
Loeken MR, Roth TF. Analysis of maternal IgG subpopulations which are transported into the chicken oocyte. Immunology. 1983; 49 :21-8.
Luckay A, Sidhu MK, Kjeken R, Megati S, Chong SY, Roopchand V, Garcia-Hand D, Abdullah R, Braun R, Montefiori DC, Rosati M, Felber BK, Pavlakis, GN, Mathiesen I, Israel ZR, Eldridge JH, Egan MA. Effect of plasmid DNA vaccine design and in vivo

electroporation on the resulting vaccine-specific immune responses in rhesus macaques. J Virol. 2007; 81:5257-9.

Luo G, Chung J, Palese P. Alterations of the stalk of the influenza virus neuraminidase: deletions and insertions. Virus Res. 1993; 29 :321.

Luschow D, Werner O, Mettenleiter TC, Fuchs W. Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. Vaccine. 2001; 19 :4249-59.

Manuguerra JC, Hannoun C. [Influenza: interspecies transmissions and viral rearrangement]. Bull Acad Natl Med. 1997; 181 :421-30.

Marangon S, Busani L, Capua I. Practicalities of the implementation of a vaccination campaign for avian influenza. Avian Dis. 2007; 51 :297-303.

Marston FA. The purification of eukaryotic polypeptides synthesized in Escherichia coli. Biochem J. 1986; 240 :1-12.

Martin K, Helenius A. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell. 1991; 67 :117-20.

Martinet W, Saelens X, Deroo T, Neirynck S, Contreras R, Min Jou W, Fiers W. Protection of mice against a lethal influenza challenge by immunization with yeast-derived recombinant influenza neuraminidase. Eur J Biochem. 1997; 247:332-8.

McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. N Engl J Med. 1983; 309 :13-27.

Meulemans G, Carlier MC, Gonze M, Petit P. Comparison of hemagglutination-inhibition, agar gel precipitin, and enzyme-linked immunosorbent assay for measuring antibodies against influenza viruses in chickens. Avian Dis. 1987; 31:560-3.

Mingxiao M, Ningyi J, Zhenguo W, Ruilin W, Dongliang F, Min Z, Gefen Y, Chang L, Leili J, Kuoshi J, Yingjiu Z. Construction and immunogenicity of recombinant fowlpox vaccines coexpressing HA of AIV H5N1 and chicken IL18. Vaccine. 2006; 24 :4304-51.

Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, Kobasa D, Kawaoka Y. Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J Virol. 2000; 74 :6015-20.

Mohan CM, Dey S, Rai A, Kataria JM. Recombinant haemagglutinin neuraminidase antigen-based single serum dilution ELISA for rapid serological profiling of Newcastle disease virus. J Virol Methods. 2006; 138 :117-20.

Montesino R, Garcia R, Quintero O, Cremata JA. Variation in N-linked oligosaccharide structures on heterologous proteins secreted by the methylotrophic yeast Pichia pastoris. Protein Expr Purif. 1998; 14 :197-207.

Morales AC, Jr, Hilt DA, Williams SM, Pantin-Jackwood MJ, Suarez DL, Spackman E, Stallknecht DE, Jackwood MW. Biologic characterization of H4, H6, and H9 type low pathogenicity avian influenza viruses from wild birds in chickens and turkeys. Avian Dis. 2009; 53 :552-62.

Murphy BR, Park EJ, Gottlieb P, Subbarao K. An influenza A live attenuated reassortant virus possessing three temperature-sensitive mutations in the PB2 polymerase gene rapidly loses temperature sensitivity following replication in hamsters. Vaccine. 1997; 15 : 1372-88.

Murphy BR, Buckler-White AJ, London WT, Snyder MH. Characterization of the M protein and nucleoprotein genes of an avian influenza A virus which are involved in host range restriction in monkeys. Vaccine. 1989; 7 :557-61.

Muster T, Subbarao EK, Enami M, Murphy BR, Palese P. An influenza A virus containing influenza B virus 5' and 3' noncoding regions on the neuraminidase gene is attenuated in mice. Proc Natl Acad Sci U S A. 1991; 15; 88 :5177-81.

Mutinelli F, Hablovarid H, Capua I. Avian embryo susceptibility to Italian H7N1 avian influenza viruses belonging to different lineages. Avian Dis. 2003; 47 :1145-9.

Nakatani H, Nakamura K, Yamamoto Y, Yamada M. Epidemiology, pathology, and immunohistochemistry of layer hens naturally affected with H5N1 highly pathogenic avian influenza in Japan. Avian Dis. 2005; 49 :436-41.

Nakaya T, Cros J, Park MS, Nakaya Y, Zheng H, Sagrera A, Villar E, Garcia-Sastre A, Palese P. Recombinant Newcastle disease virus as a vaccine vector. J Virol. 2001; 75 :11868-73.

Nayak B, Rout SN, Kumar S, Khalil MS, Fouda MM, Ahmed LE, Earhart KC, Perez DR, Collins PL, Samal SK. Immunization of chickens with Newcastle disease virus expressing H5 hemagglutinin protects against highly pathogenic H5N1 avian influenza viruses. PLoS One. 2009; 4 :6509.

Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med. 1999; 5 :1157-63.

Nesterenko MV, Tilley M, Upton SJ. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. J Biochem Biophys Methods. 1994; 28 :239-42.

Neumann G, Hatta M, Kawaoka Y. Reverse genetics for the control of avian influenza. Avian Dis. 2003; 47 :882-7.

Normile D. Avian influenza. China will attempt largest-ever animal vaccination campaign. Science. 2005; 310 :1256-7.

Nwe N, He Q, Damrongwatanapokin S, Du Q, Manopo I, Limlamthong Y, Fenner BJ, Spencer L, Kwang J. Expression of hemagglutinin protein from the avian influenza virus H5N1 in a baculovirus/insect cell system significantly enhanced by suspension culture. BMC Microbiol. 2006; 6 :16.

Ohuchi R, Ohuchi M, Garten W, Klenk HD. Oligosaccharides in the stem region maintain the influenza virus hemagglutinin in the metastable form required for fusion activity. J Virol. 1997; 71 :3719-25.

OIE, 2005, Manual of diagnostic tests and vaccines for terrestrial animals. Chapter, 2.7.12., Version adapted may, 2005.

Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science. 2006; 312 :384-8.

Olsen CW. DNA vaccination against influenza viruses: a review with emphasis on equine and swine influenza. Vet Microbiol. 2000; 74 :149-64.

Omar AR, Kim CL, Bejo MH, Ideris A. Efficacy of VP2 protein expressed in E. coli for protection against highly virulent infectious bursal disease virus. J Vet Sci. 2006; 7 :241-7.

Orr-Weaver TL, Szostak JW, Rothstein RJ. Yeast transformation: a model system for the study of recombination. Proc Natl Acad Sci U S A. 1981; 78 :6354-8.

Osterholm MT. Preparing for the next pandemic. N Engl J Med. 2005: 352 :1839-42.

Pan Z, Zhang X, Geng S, Cheng N, Sun L, Liu B, Huang J, Jiao X. Priming with a DNA vaccine delivered by attenuated Salmonella typhimurium and boosting with a killed vaccine confers protection of chickens against infection with the H9 subtype of avian influenza virus. Vaccine. 2009; 27 :1018-23.

Pantin-Jackwood MJ, Swayne DE. Pathogenesis and pathobiology of avian influenza virus infection in birds. Rev Sci Tech. 2009; 28 :113-36.

Park MS, Steel J, Garcia-Sastre A, Swayne D, Palese P. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. Proc Natl Acad Sci U S A. 2006; 103 :8203-8.

Park YW, Katze MG. Translational control by influenza virus. Identification of cis-acting sequences and trans-acting factors which may regulate selective viral mRNA translation. J Biol Chem. 1995; 270 :28433-9.

Pasick J, Handel K, Robinson J, Copps J, Ridd D, Hills K, Kehler H, Cottam-Birt C, Neufeld J, Berhane Y, Czub S. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J General Virology. 2005; 86 :727 -31.

Pavlova SP, Veits J, Keil GM, Mettenleiter TC, Fuchs W. Protection of chickens against H5N1 highly pathogenic avian influenza virus infection by live vaccination with infectious laryngotracheitis virus recombinants expressing H5 hemagglutinin and N1 neuraminidase. Vaccine. 2009a; 27 :773-85.

Pavlova SP, Veits J, Mettenleiter TC, Fuchs W. Live vaccination with an H5-hemagglutinin-expressing infectious laryngotracheitis virus recombinant protects chickens against different highly pathogenic avian influenza viruses of the H5 subtype. Vaccine. 2009b; 27 :5085-90.

Payungporn S, Phakdeewirot P, Chutinimitkul S, Theamboonlers A, Keawcharoen J, Oraveerakul K, Amonsin A, Poovorawan Y. Single-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) for influenza A virus subtype H5N1 detection. Viral Immunol. 2004;17 :588-93.

Peng JM, Tong GZ, Wang YF, Qiu HJ. Multi-epitope DNA vaccines against avian influenza in chickens. Sheng Wu Gong Cheng Xue Bao. 2003; 19 :623-7.

Perez-Ramirez E, Rodriguez V, Sommer D, Blanco JM, Acevedo P, Heffels-Redmann U, Hofle U. Serologic testing for avian influenza viruses in wild birds: comparison of two commercial competition enzyme-linked immunosorbent assays. Avian Dis. 2010; 54 :729-33.

Perez DR, Donis RO. The matrix 1 protein of influenza A virus inhibits the transcriptase activity of a model influenza reporter genome in vivo. Virology. 1998; 249 :52-61.

Perez DR, Sorrell EM, Donis RO. Avian influenza: an omnipresent pandemic threat. Pediatr Infect Dis J. 2005; 24: 208-16.

Philippa JD, Munster VJ, Bolhuis H, Bestebroer TM, Schaftenaar W, Beyer WE, Fouchier RA, Kuiken T, Osterhaus AD. Highly pathogenic avian influenza (H7N7): vaccination of zoo birds and transmission to non-poultry species. Vaccine. 2005; 23 :5743-50.

Philpott M, Easterday BC, Hinshaw VS. Neutralizing epitopes of the H5 hemagglutinin from a virulent avian influenza virus and their relationship to pathogenicity. J Virol. 1989a; 63 :3453-8.

Philpott M, Hioe C, Sheerar M, Hinshaw VS. Hemagglutinin mutations related to attenuation and altered cell tropism of a virulent avian influenza A virus. J Virol. 1990; 64 :2941-7.

Philpott MS, Easterday BC, Hinshaw VS. Antigenic and phenotypic variants of a virulent avian influenza virus selected during replication in ducks. J Wildl Dis. 1989b; 25 :507-13.

Phipps LP, Essen SC, Brown IH. Genetic subtyping of influenza A viruses using RT-PCR with a single set of primers based on conserved sequences within the HA2 coding region. J Virol Methods. 2004; 122 :119-22.

Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. Cell. 1992; 69 :517-28.

Polson A, von Wechmar MB, van Regenmortel MH. Isolation of viral IgY antibodies from yolks of immunized hens. Immunol Commun. 1980; 9 :475-93.

Portela A, Digard P. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J Gen Virol. 2002; 83 :723-34.

Portocarrero C, Markley K, Koprowski H, Spitsin S, Golovkin M. Immunogenic properties of plant-derived recombinant smallpox vaccine candidate pB5. Vaccine. 2008; 26 :5535-40.

Possee RD. Cell-surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. Virus Res. 1986; 5 :43-59.

Powers DC, Smith GE, Anderson EL, Kennedy DJ, Hackett CS, Wilkinson BE, Volvovitz F, Belshe RB, Treanor JJ. Influenza A virus vaccines containing purified recombinant H3 hemagglutinin are well tolerated and induce protective immune responses in healthy adults. J Infect Dis. 1995; 171:1595-9.

Prabakaran M, Ho HT, Prabhu N, Velumani S, Szyporta M, He F, Chan KP, Chen LM, Matsuoka Y, Donis RO, Kwang J. Development of epitope-blocking ELISA for

universal detection of antibodies to human H5N1 influenza viruses. PLoS One. 2009; 4 :4566.

Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, Khanyok R, Thawachsupa P, Kijphati R, Sawanpanyalert P. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol. 2005; 86:423-33.

Qiao C, Jiang Y, Tian G, Wang X, Li C, Xin X, Chen H, Yu K. Recombinant fowlpox virus vector-based vaccine completely protects chickens from H5N1 avian influenza virus. Antiviral Res. 2009; 81 :234-8.

Raj GD, Jones RC. Local antibody production in the oviduct and gut of hens infected with a variant strain of infectious bronchitis virus. Vet Immunol Immunopathol. 1996; 53 :147-61.

Rao S, Kong WP, Wei CJ, Yang ZY, Nason M, Styles D, DeTolla LJ, Panda A, Sorrell EM, Song H, Wan H, Ramirez-Nieto GC, Perez D, Nabel GJ. Multivalent HA DNA vaccination protects against highly pathogenic H5N1 avian influenza infection in chickens and mice. PLoS One. 2008; 3 :2432.

Raviprakash K, Porter KR. Needle-free injection of DNA vaccines: a brief overview and methodology. Methods Mol Med. 2006; 127 :83-9.

Reed LJ, and Muench H. A simple method of estimating fifty percent endpoints, *Am. J. Hyg.* 1938; 27 :493–7.

Robinson HL, Boyle CA, Feltquate DM, Morin MJ, Santoro JC, Webster RG. DNA immunization for influenza virus: studies using hemagglutinin- and nucleoprotein-expressing DNAs. J Infect Dis. 1997; 176 :50-5.

Romanos MA, Scorer CA, Clare JJ. Foreign gene expression in yeast: a review. Yeast. 1992; 8 :423-88.

Romer-Oberdorfer A, Veits J, Helferich D, Mettenleiter TC. Level of protection of chickens against highly pathogenic H5 avian influenza virus with Newcastle disease virus based live attenuated vector vaccine depends on homology of H5 sequence between vaccine and challenge virus. Vaccine. 2008; 26 :2307-13.

Rose ME, Orlans E, Buttress N. Immunoglobulin classes in the hen's egg: their segregation in yolk and white. Eur J Immunol. 1974; 4 :521-3.

Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, Fukuda K, Cox NJ, Katz JM. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol. 1999; 37 :937-43.

Saelens X, Vanlandschoot P, Martinet W, Maras M, Neirynck S, Contreras R, Fiers W, Jou WM. Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. Eur J Biochem. 1999; 260 :166-75.

Sagt CM, Kleizen B, Verwaal R, de Jong MD, Muller WH, Smits A, Visser C, Boonstra J, Verkleij AJ, Verrips CT. Introduction of an N-glycosylation site increases secretion of heterologous proteins in yeasts. Appl Environ Microbiol. 2000; 66 :4940-4.

Sainz-Pastor N, Tolner B Huhalov A, Kogelberg H, Lee YC, Zhu D, Begent RH, Chester KA. Deglycosylation to obtain stable and homogeneous Pichia pastoris-expressed N-A1 domains of carcinoembryonic antigen. Int J Biol Macromol. 2006; 39 :141-50.

Sarukhan A, Camugli S, Gjata B, von Boehmer H, Danos O, Jooss K. Successful interference with cellular immune responses to immunogenic proteins encoded by recombinant viral vectors. J Virol. 2001; 75 :269-77.

Scanlan CN, Ritchie GE, Baruah K, Crispin M, Harvey DJ, Singer BB, Lucka L, Wormald MR, Wentworth P, Jr, Zitzmann N, Rudd PM, Burton DR, Dwek RA. Inhibition of mammalian glycan biosynthesis produces non-self antigens for a broadly neutralising, HIV-1 specific antibody. J Mol Biol. 2007; 372 :16-22.

Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, Ott RG, Anthony RM, Zebroski H, Hurley A, Phogat A, Chakrabarti B, Li Y, Connors M, Pereyra F, Walker BD, Wardemann H, Ho D, Wyatt RT, Mascola JR, Ravetch JV, Nussenzweig MC. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. Nature. 2009; 458 :636-40.

Schroer D, Veits J, Grund C, Dauber M, Keil G, Granzow H, Mettenleiter TC, Romer-Oberdorfer A. Vaccination with Newcastle disease virus vectored vaccine protects chickens against highly pathogenic H7 avian influenza virus. Avian Dis. 2009; 53 :190-7.

Selleck PW, Lowther SL, Russell GM, Hooper PT. Rapid diagnosis of highly pathogenic avian influenza using pancreatic impression smears. Avian Dis. 2003 ;47 :1190-5.

Sellers H, Linneman E, Icard AH, Mundt E. A purified recombinant baculovirus expressed capsid protein of a new astrovirus provides partial protection to runting-stunting syndrome in chickens. Vaccine. 2010; 28 :1253-63.

Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Suss J, Lipkind M, Kida H, Webster RG. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis. 1996; 40 :425-37.

Seo SH, Goloubeva O, Webby R, Webster RG. Characterization of a porcine lung epithelial cell line suitable for influenza virus studies. J Virol. 2001; 75 :9517-25.

Seo SH, Peiris M, Webster RG. Protective cross-reactive cellular immunity to lethal A/Goose/Guangdong/1/96-like H5N1 influenza virus is correlated with the proportion of pulmonary CD8(+) T cells expressing gamma interferon. J Virol. 2002; 76 :4886-90.

Sha B, Luo M. Crystallization and preliminary X-ray crystallographic studies of type A influenza virus matrix protein M1. Acta Crystallogr D Biol Crystallogr. 1997; 53 :458-60.

Sharon N, Lis H. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology. 2004; 14 :53-62.

Shoham D. Review: molecular evolution and the feasibility of an avian influenza virus becoming a pandemic strain--a conceptual shift. Virus Genes. 2006; 33 :127-32.

Shoji Y, Chichester JA, Bi H, Musiychuk K, de la Rosa P, Goldschmidt L, Horsey A, Ugulava N, Palmer GA, Mett V, Yusibov V. Plant-expressed HA as a seasonal influenza vaccine candidate. Vaccine. 2008; 26 :2930-4.

Shortridge KF. Avian influenza A viruses of southern China and Hong Kong: ecological aspects and implications for man. Bull World Health Organ. 1982; 60 :129-35.

Sidorenko Y, Reichl U. Structured model of influenza virus replication in MDCK cells. Biotechnol Bioeng. 2004; 88 :1-14.

Sieczkarski SB, Whittaker GR. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J Virol. 2002; 76 :10455-64.

Singh I, Doms RW, Wagner KR, Helenius A. Intracellular transport of soluble and membrane-bound glycoproteins: folding, assembly and secretion of anchor-free influenza hemagglutinin. EMBO J. 1990; 9 :631-9.

Skehel JJ, Bayley PM, Brown EB, Martin SR, Waterfield MD, White JM, Wilson IA, Wiley DC. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc Natl Acad Sci U S A. 1982; 79 :968-72.

Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem. 2000; 69 :531-69.

Slepushkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. Vaccine. 1995;13 :1399-402.

Smeenk CA, Brown EG. The influenza virus variant A/FM/1/47-MA possesses single amino acid replacements in the hemagglutinin, controlling virulence, and in the matrix protein, controlling virulence as well as growth. J Virol. 1994; 68 :530-4.

Snyder DB, Marquardt WW, Yancey FS, Savage PK. An enzyme-linked immunosorbent assay for the detection of antibody against avian influenza virus. Avian Dis. 1985; 29 :136-44.

Spitsin S, Andrianov V, Pogrebnyak N, Smirnov Y, Borisjuk N, Portocarrero C, Veguilla V, Koprowski H, Golovkin M. Immunological assessment of plant-derived avian flu H5/HA1 variants. Vaccine, 2009; 27 :1289-92.

Steel J, Burmakina SV, Thomas C, Spackman E, Garcia-Sastre A, Swayne DE, Palese P. A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine. 2008; 26 :522-31.

Steensels M, Bublot M, Van Borm S, De Vriese J, Lambrecht B, Richard-Mazet A, Chanavat-Bizzini S, Duboeuf M, Le Gros FX, van den Berg T. Prime-boost vaccination with a fowlpox vector and an inactivated avian influenza vaccine is highly immunogenic in Pekin ducks challenged with Asian H5N1 HPAI. Vaccine. 2009; 27 :646-54.

Stephenson I, Heath A, Major D, Newman RW, Hoschler K, Junzi W, Katz JM, Weir JP, Zambon MC, Wood JM. Reproducibility of serologic assays for influenza virus A (H5N1). Emerg Infect Dis. 2009; 15 :1252-9.

Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science. 2006; 312 :404-10.

Stieneke-Grober A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. 1992; 11 :2407-14.

Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, Buranathai C, Nguyen TD, Chaisingh A, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG. Are ducks contributing to the

endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol. 2005; 79: 11269-79.

Suarez DL. Overview of avian influenza DIVA test strategies. Biologicals. 2005; 33 :221-6.

Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, Swayne DE. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol. 1998; 72 :6678-88.

Suarez DL, Schultz-Cherry S. Immunology of avian influenza virus: a review. Dev Comp Immunol. 2000; 24 :269-83.

Subbarao K, Joseph T. Scientific barriers to developing vaccines against avian influenza viruses. Nat Rev Immunol. 2007; 7 :267-78.

Sugiura T, Sugita S, Imagawa H, Kanaya T, Ishiyama S, Saeki N, Uchiyama A, Tanigawa M, Kuwano A. Serological diagnosis of equine influenza using the hemagglutinin protein produced in a baculovirus expression system. J Virol Methods. 2001; 98 :1-8.

Sugrue RJ, Hay AJ. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology. 1991; 180 :617-24.

Suguitan AL Jr, Marino MP, Desai PD, Chen LM, Matsuoka Y, Donis RO, Jin H, Swayne DE, Kemble G, Subbarao K. The influence of the multi-basic cleavage site of the H5 hemagglutinin on the attenuation, immunogenicity and efficacy of a live attenuated influenza A H5N1 cold-adapted vaccine virus. Virology. 2009; 20;395 :280-8.

Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol. 2009; 16 :265-73.

Suzuki K, Okada H, Itoh T, Tada T, Tsukamoto K. Phenotypes influencing transmissibility of highly pathogenic avian influenza viruses in chickens. J Gen Virol. 2010.

Swayne DE. Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1 high pathogenicity avian influenza. Ann N Y Acad Sci. 2006a; 1081 :174-81.

Swayne DE. Recommendations from the avian influenza vaccine workshop. Ann N Y Acad Sci. 2006b; 1081 :226-7.

Swayne DE. Avian influenza vaccines and therapies for poultry. Comp Immunol Microbiol Infect Dis. 2009; 32 :351-63.

Swayne DE, Beck JR. Experimental study to determine if low-pathogenicity and high-pathogenicity avian influenza viruses can be present in chicken breast and thigh meat following intranasal virus inoculation. Avian Dis. 2005; 49:81-5.

Swayne DE, Beck JR, Kinney N. Failure of a recombinant fowl poxvirus vaccine containing an avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowl pox vaccine. Avian Dis. 2000; 44 :132-7.

Swayne DE, Beck JR, Perdue ML, Beard CW. Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza. Avian Dis. 2001; 45 :355-65.

Swayne DE, Kapczynski D. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunol Rev. 2008; 225 :314-31.

Swayne DE, Perdue ML, Garcia M, Rivera-Cruz E, Brugh M. Pathogenicity and diagnosis of H5N2 Mexican avian influenza viruses in chickens. Avian Dis. 1997; 41 :335-46.

Swayne DE, Suarez DL. Highly pathogenic avian influenza. Rev Sci Tech. 2000; 19 :463-82.

Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Jpn J Infect Dis. 2005; 58 :195-207.

Tang Y, Wu P, Peng D, Wang X, Wan H, Zhang P, Long J, Zhang W, Li Y, Wang W., Zhang X, Liu X. Characterization of duck H5N1 influenza viruses with differing pathogenicity in mallard (Anas platyrhynchos) ducks. Avian Pathol. 2009; 38 :457-67.

Taylor J, Weinberg R, Kawaoka Y, Webster RG, Paoletti E. Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine. 1988; 6 :504-8.

Tian G, Zhang S, Li Y, Bu Z, Liu P, Zhou J, Li C, Shi J, Yu K, Chen H. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virology. 2005; 341 :153-62.

Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, Hogan RJ, Wu Z, Benton KA, Tumpey TM, Epstein SL. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg Infect Dis. 2007; 13 :426-35.

Tonegawa K, Nobusawa E, Nakajima K, Kato T, Kutsuna T, Kuroda K, Shibata T, Harada Y, Nakamura A, Itoh M. Analysis of epitope recognition of antibodies induced by DNA immunization against hemagglutinin protein of influenza A virus. Vaccine. 2003; 21 :3118-25.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979; 76 :4350-4.

Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med. 2006; 354 : 1343-51.

Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D, Wolff M, Rabinovich G, Blackwelder W, Katz JM. Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Vaccine. 2001; 19 :1732-7.

Tsuchiya E, Sugawara K, Hongo S, Matsuzaki Y, Muraki Y, Li ZN, Nakamura K. Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus. J Gen Virol. 2001; 82 :2475-84.

Tsumoto K, Ejima D, Kumagai I, Arakawa T. Practical considerations in refolding proteins from inclusion bodies. Protein Expr Purif. 2003; 28 :1-8.

Tumpey TM, Alvarez R, Swayne DE, Suarez DL. Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. J Clin Microbiol. 2005; 43 :676-83.

Upadhyay C, Ammayappan A, Vakharia VN. Detection of NP, N3 and N7 antibodies to avian influenza virus by indirect ELISA using yeast-expressed antigens. Virol J. 2009; 6 :158.

van der Goot, J.A., Koch, G., de Jong, M.C., van Boven, M., 2005, Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. Proc Natl Acad Sci U S A 102 :18141-6.

Veits J, Romer-Oberdorfer A, Helferich D, Durban M, Suezer Y, Sutter G, Mettenleiter TC. Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions. Vaccine. 2008; 26 :1688-96.

Veits J, Wiesner D, Fuchs W, Hoffmann B, Granzow H, Starick E, Mundt E, Schirrmeier H, Mebatsion T, Mettenleiter TC, Romer-Oberdorfer A. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. Proc Natl Acad Sci U S A. 2006; 103 :8197-202.

Vey M, Orlich M, Adler S, Klenk HD, Rott R, Garten W. Hemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif R-X-K/R-R. Virology. 1992; 188 :408-13.

Vigerust DJ, Ulett KB, Boyd KL, Madsen J, Hawgood S, McCullers JA. N-linked glycosylation attenuates H3N2 influenza viruses. J Virol. 2007; 81 :8593-600.

Vignuzzi M, Gerbaud S, van der Werf S, Escriou N. Naked RNA immunization with replicons derived from poliovirus and Semliki Forest virus genomes for the generation of a cytotoxic T cell response against the influenza A virus nucleoprotein. J Gen Virol. 2001; 82 :1737-47.

Wagner R, Heuer D, Wolff T, Herwig A, Klenk HD. N-Glycans attached to the stem domain of haemagglutinin efficiently regulate influenza A virus replication. J Gen Virol. 2002; 83 :601-9.

Wakefield L, Brownlee GG. RNA-binding properties of influenza A virus matrix protein M1. Nucleic Acids Res. 1989; 17 :8569-80.

Wang C, Lamb RA, Pinto LH. Direct measurement of the influenza A virus M2 protein ion channel activity in mammalian cells. Virology. 1994; 205 :133-40.

Wang G, Hu S, Yu X. Development of a latex agglutination test for detecting antibodies against avian influenza virus based on matrix 1 protein expressed in vitro. Avian Dis. 2010; 54 :41-5.

Wang K, Holtz KM, Anderson K, Chubet R, Mahmoud W, Cox MM. Expression and purification of an influenza hemagglutinin--one step closer to a recombinant protein-based influenza vaccine. Vaccine. 2006; 24 :2176-85.

Webby RJ, Webster RG. Are we ready for pandemic influenza? Science. 2003; 302 : 1519-22.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992; 56 :152-79.

Webster RG, Webby RJ, Hoffmann E, Rodenberg J, Kumar M, Chu HJ, Seiler P, Krauss S, Songserm T. The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. Virology. 2006; 351 :303-11.

Wei CJ, Xu L, Kong WP, Shi W, Canis K, Stevens J, Yang ZY, Dell A, Haslam SM, Wilson IA, Nabel GJ. Comparative efficacy of neutralizing antibodies elicited by recombinant hemagglutinin proteins from avian H5N1 influenza virus. J Virol. 2008; 82 :6200-8.

Weidner M, Taupp M, Hallam SJ. Expression of recombinant proteins in the methylotrophic yeast Pichia pastoris. J Vis Exp. 2010.

Weller CT, Lustbader J, Seshadri K, Brown JM, Chadwick CA, Kolthoff CE, Ramnarain S, Pollak S, Canfield R, Homans SW. Structural and conformational analysis of glycan moieties in situ on isotopically 13C, 15N-enriched recombinant human chorionic gonadotropin. Biochemistry. 1996; 35 :8815-23.

White J, Kartenbeck J, Helenius A. Membrane fusion activity of influenza virus. EMBO J. 1982; 1 :217-22.

Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu Rev Biochem. 1987; 56 :365-94.

Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature. 1981; 289 :373-8.

Wilson BS, Glassy MC, Quaranta V, Ng AK, Ferrone S. Effect of tunicamycin on the assembly and antigenicity of HLA antigens: analysis with monoclonal antibodies. Scand J Immunol. 1981a; 14 :201-5.

Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature. 1981b; 289 :366-373.

Wood GW, McCauley JW, Bashiruddin JB, Alexander DJ. Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Arch Virol. 1993; 130 :209-17.

Woolcock PR, McFarland MD, Lai S, Chin RP. Enhanced recovery of avian influenza virus isolates by a combination of chicken embryo inoculation methods. Avian Dis. 2001; 45 :1030-5.

Wu H, Scissum-Gunn K, Singh NK, Giambrone JJ. Toward the development of a plant-based vaccine against reovirus. Avian Dis. 2009; 53 :376-81.

Xie QM, Ji J, Du LQ, Cao YC, Wei L, Xue CY, Qin JP, Ma JY, Bi YZ. Preparation and immune activity analysis of H5N1 subtype avian influenza virus recombinant protein-based vaccine. Poult Sci. 2009; 88 :1608-15.

Yang T, Wang HN, Wang X, Tang JN, Lu D, Zhang YF, Guo ZC, Li YL, Gao R, Kang RM. The protective immune response against infecs bronchitis virus induced by multi-epitope based peptide vaccines. Biosci Biotechnol Biochem. 2009; 73 :1500-4.

Yang ZY, Wei CJ, Kong WP, Wu L, Xu L, Smith DF, Nabel GJ. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. Science. 2007; 317 :825-8.

Yasuda J, Bucher DJ, Ishihama A. 1994, Growth control of influenza A virus by M1 protein: analysis of transfectant viruses carrying the chimeric M gene. J Virol. 1994; 68 :8141-6.

Ye Z, Liu T, Offringa DP, McInnis J, Levandowski RA. Association of influenza virus matrix protein with ribonucleoproteins. J Virol. 1999; 73 :7467-73.

Ye ZP, Baylor NW, Wagner RR. Transcription-inhibition and RNA-binding domains of influenza A virus matrix protein mapped with anti-idiotypic antibodies and synthetic peptides. J Virol. 1989; 63 :3586-94.

Yewdell JW, Bennink JR, Smith GL, Moss B. 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. 1985; 82 :1785-9.

Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J Virol. 1988; 62 :2762-72.

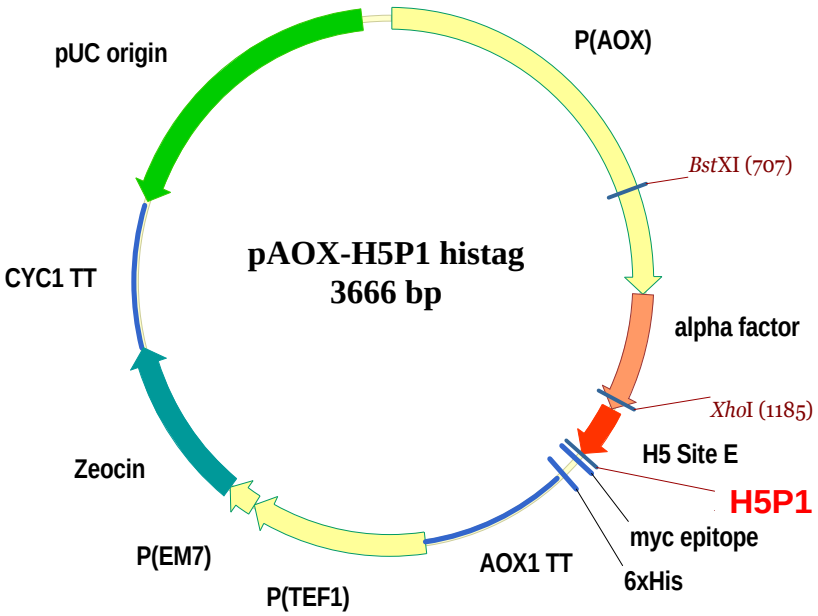
Zheng L, Wang F, Yang Z, Chen J, Chang H, Chen Z. A single immunization with HA DNA vaccine by electroporation induces early protection against H5N1 avian influenza virus challenge in mice. BMC Infect Dis. 2009; 9 :17.

Zhou EM, Chan M, Heckert RA, Riva J, Cantin MF. Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. Avian Dis. 1998; 42 :517-22.

Zhu A, Wang ZK, Beavis R. Structural studies of alpha-N-acetylgalactosaminidase: effect of glycosylation on the level of expression, secretion efficiency, and enzyme activity. Arch Biochem Biophys. 1998; 352 :1-8.

Zvonarjev AY, Ghendon YZ. Influence of membrane (M) protein on influenza A virus virion transcriptase activity in vitro and its susceptibility to rimantadine. J Virol. 1980; 33 :583-6.

APPENDIX



The coding sequence of P1 polypeptide cloned to pAOX vector.

P1 – sequence including alpha factor, gene of interest and histag

<u>**M** R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I P A E A V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I N T T I A S I A A K E E G V S L E K R E A E A</u> **D L D G V K P L I L R D C S V A G W L L G N P M C D E F I N V P E W S Y I V E K** *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Alpha Factor: underlined

P1 sequence: bold

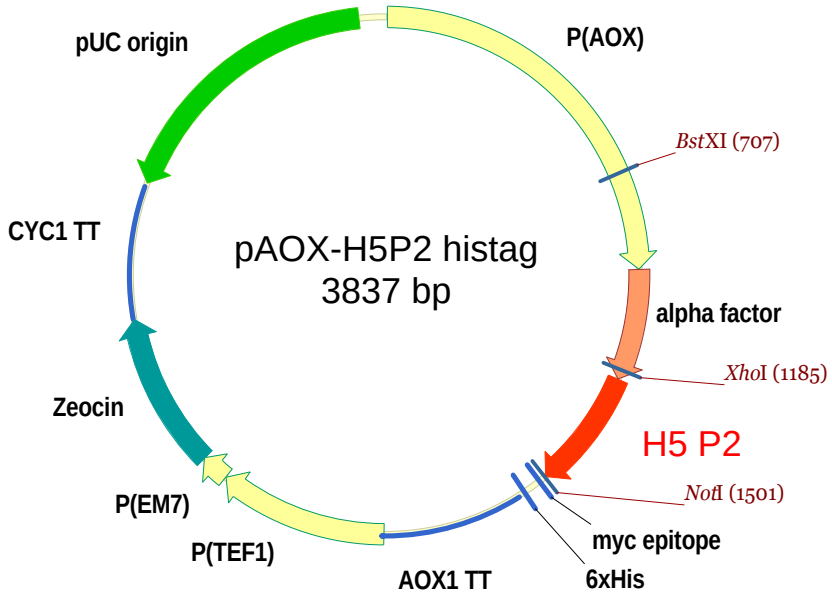
His tag and remains of MCS: Italic

Theoretical pI / Mw: 4.40 / 16912.92 (Da)

P1 polypeptide when it is completely processed

D L D G V K P L I L R D C S V A G W L L G N P M C D E F I N V P E W S Y I V E K *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Theoretical pI / Mw: 4.88 / 7591.53



The coding sequence of P2 polypeptide cloned to pAOX vector.

P2 – sequence including alpha factor, gene of interest and histag

M R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I P A E A V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I N T T I A S I A A K E E G V S L E K R E A E A **N N T N Q E D L L V L W G I H H P N D A A E Q T K L Y Q N P T T Y I S V G T S T L N Q R L V P R I A T R S K V N G Q S G R M E F F W T I L K P N D A I N F E S N G N F I A P E Y A Y K I V K K G** *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Alpha Factor: underlined

P2 sequence: bold

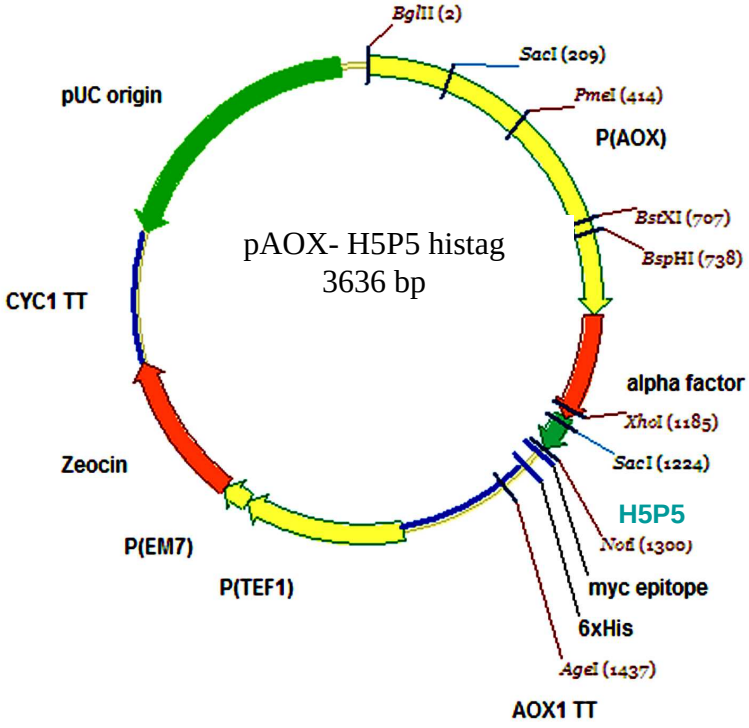
His tag and remains of MCS: Italic

Theoretical pI / Mw: 5.09 / 23272.92 (Da)

P2 polypeptide when it is completely processed

N N T N Q E D L L V L W G I H H P N D A A E Q T K L Y Q N P T T Y I S V G T S T L N Q R L V P R I A T R S K V N G Q S G R M E F F W T I L K P N D A I N F E S N G N F I A P E Y A Y K I V K K G *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Theoretical pI / Mw: 6.46 / 13951.53 (Da)



The coding sequence of P5 polypeptide cloned to pAOX vector.

P5- sequence including alpha-factor, gen of interest and his-tag

M R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I P A E A V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I N T T I A S I A A K E E G V S L E K R E A E A **S L G V S S A C P Y Q R K S S F F R N V V W L I K K N S T Y** *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Alpha Factor: underlined

P5 sequence: bold

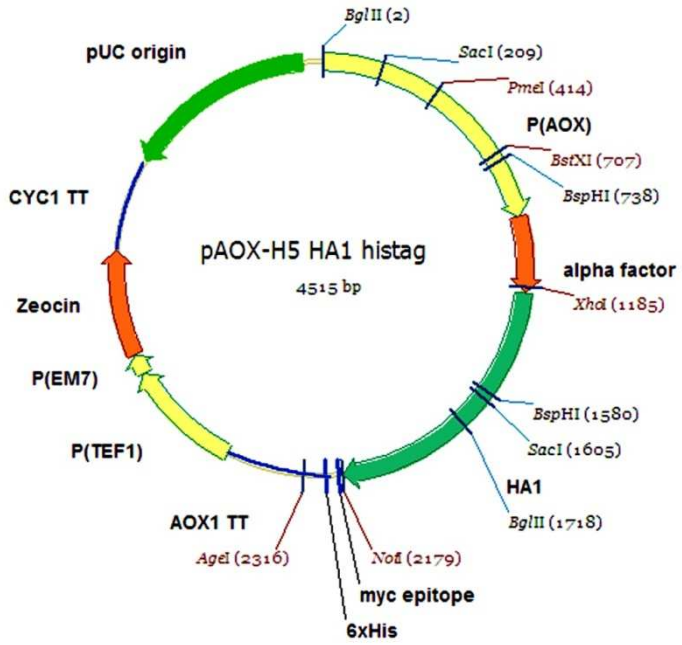
His tag and remains of MCS: Italic

Theoretical pI / Mw: 5.10 /15843.67 (Da)

P5 polypeptide when it is completely processed

S L G V S S A C P Y Q R K S S F F R N V V W L I K K N S T Y *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Theoretical pI / Mw: 7.98/ 6522.28 (Da)



The coding sequence of rHA1- polypeptide cloned to pAOX vector.

HA1- sequence including alpha-factor, gen of interest and histag

M R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I P A E A V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I N T T I A S I A A K E E G V S L E K R E A E A **D Q I C I G Y H A N N S T E Q V D T I M E K N V T V T H A Q D I L E K T H N G K L C D L D G V K P L I L R D C S V A G W L L G N P M C D E F I N V P E W S Y I V E K A N P V N D L C Y P G D F N D Y E E L K H L L S R I N H F E K I Q I I P K S S W S S H E A S L G V S S A C P Y Q R K S S F F R N V V W L I K K N S T Y P T I K R S Y N N T N Q E D L L V L W G I H H P N D A A E Q T K L Y Q N P T T Y I S V G T S T L N Q R L V P R I A T R S K V N G Q S G R M E F F W T I L K P N D A I N F E S N G N F I A P E Y A Y K I V K K G D S T I M K S E L E Y G N C N T K C Q T P M G A I N S S M P F H N I H P L T I G E C P K Y V K S N R L V L A T G L R N S P Q R** *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Theoretical pI / Mw: 5.82 / 48996.22

HA1- polypeptide when it is completely processed

D Q I C I G Y H A N N S T E Q V D T I M E K N V T V T H A Q D I L E K T H N G K L C D L D G V K P L I L R D C S V A G W L L G N P M C D E F I N V P E W S Y I V E K A N P V N D L C Y P G D F N D Y E E L K H L L S R I N H F E K I Q I I P K S S W S S H E A S L G V S S A C P Y Q R K S S F F R N V V W L I K K N S T Y P T I K R S Y N N T N Q E D L L V L W G I H H P N D A A E Q T K L Y Q N P T T Y I S V G T S T L N Q R L V P R I A T R S K V N G Q S G R M E F F W T I L K P N D A I N F E S N G N F I A P E Y A Y K I V K K G D S T I M K S E L E Y G N C N T K C Q T P M G A I N S S M P F H N I H P L T I G E C P K Y V K S N R L V L A T G L R N S P Q R *A A A S F L E Q K L I S E E D L N S A V D H H H H H H*

Theoretical p I/ Mw: 6.80 / 39674.8

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