AEROSOL VACCINATION OF CHICKENS WITH BACULOVIRUS EXPRESSED VIRUS-LIKE PARTICLES INDUCED IMMUNE RESPONSE IN CHICKENS

by

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Under the Direction of Egbert Mundt

ABSTRACT

Influenza A virus (IAV) vaccination of animals and humans is a powerful tool for prevention and control of disease. Currently licensed vaccines are egg-based and delivered by injection. Baculovirus grown in insect cells cultures can produce high yields of virus-like particles (VLP). VLP vaccines have been shown to be highly immunogenic after parenteral application in mice, ferrets, and humans. The aim of this study was to assess influenza VLPs, as an aerosolized vaccine, as a vaccination strategy in chickens. One-day-old SPF chickens were vaccinated twice with 100 μg, 20 μg, or 5 μg of VLPs. As control, chickens were also vaccinated via intranasal instillation and intramuscular injection. The VLPs induced seroconversion after intramuscular application at any dosage. In contrast, aerolized VLPs induced a specific antibody response after aerosolization but only when 100 μg were given. These data show that influenza VLPs might be used for mass aerosol vaccination in chickens.

INDEX WORDS: Influenza virus, Virus-like particle, Aerosol, Mass vaccination

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DEDICATION

I would like to dedicate this thesis to my friends and family who have helped me throughout my time as a graduate student. I would especially like to thank my parents, David and Margaret Earnest, for providing more support, advice, and love than I ever deserved. It is because of you that I am where I am. I like to thank my grandparents for the support they have given to me throughout my education. I would also like to thank my friends in Athens and throughout the country for keeping me grounded and making the last two years great for me.

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CHAPTER 1

INTRODUCTION

The described experiments were performed to determine if the vaccination of chickens with aerosolized virus-like particles (VLPs) can induce an immune response in naive birds. Currently available influenza virus vaccines are not ideal for use in poultry due to the fact that they have to be administered on an individual basis either subcutaneously or intramuscularly. The current production methods for poultry is based on industrial methods. This means that a large number of chickens are produced under very intense conditions and methods for mass vaccination of chickens against avian influenza are needed but not available. To substantiate this need, the USA produced approximately 8 billion broilers and 90 billion table eggs in 2010 (http://www.poultryegg.org/economic_data/). With a risk to the poultry industry posed by avian influenza viruses of either hemagglutinin 5 (HA5) or HA7 subtypes and the possibility of widespread pandemics of highly pathogenic avian influenza viruses (HPAI), it is important for the poultry industry to develop novel methods for immunizing and protecting flocks. Using virus-like particles may allow influenza vaccines to be produced quickly, specifically, and cheaply. Being able to deliver these vaccines using an aerosol route will allow for the vaccines to be used on much larger numbers of birds.

Influenza virus-like particles have been shown to induce a protective immune response in mammalian species. However, this has not been previously shown in avian species. Furthermore, there is no influenza vaccine available at this time that can be delivered to poultry flocks via an aerosol. This research was performed to investigate if influenza A virus-based

VLPs can induce seroconversion in naive chickens when delivered via aerosol. To investigate this, birds were exposed to controlled amounts of aerosolized influenza VLPs. In parallel, birds were vaccinated with VLPs via the intramuscular and intranasal routes. The presence of influenza specific serum antibodies was tested by means of hemagglutination inhibition assays, enzyme-linked immunosorbent assays (ELISA), and an indirect ELISA to confirm seroconversion.

The hypothesis was that that when chickens are given influenza virus-like particles via an aerosol route, the exposed birds will produce antibodies against the hemagglutinin protein. The research will help to clarify if exposure to influenza A virus based VLPs has the ability to be used as aerosol-based vaccine. This would be a viable approach to vaccinate chickens using a mass application system.

CHAPTER 2

LITERATURE REVIEW

History and Clinical Disease of Influenza A Virus Infection

Influenza viruses has been theorized to have been infecting avian and mammalian species since the times of ancient Greek writings. There is a great amount of evidence for at least 10 global pandemics since 1590 AD (1). The virus was first characterized as "the fowl plague" by an Italian named Perroncito in 1878. Perroncito described a disease that resulted in a contagious and highly fatal disease that spread among poultry in northern Italy (2). The virus causing this disease belonged to the family *Orthomyxoviridae*. These viruses contain a segmented, single-stranded RNA genome of negative sense orientation. *Orthomyxoviridae* has been divided into *Influenzaviruses A, Influenzaviruses B, Influenzaviruses C, Thogotovirus, and Isavirus* (3). Viruses of the genus influenza A virus are able to infect a wide array of animals. Wild birds are the natural reservoir hosts for influenza A virus but they mostly show no signs of clinical disease when infected (4,5) Influenza A virus can also infect mammals including humans, dogs, cats, horses, swine, and even sea mammals. Influenza viruses can cause disease with a high variability in the clinical outcome depending on viral and host factors, however they tend to be asymptomatic in the wild bird reservoirs.

After the description of "fowl plague" by Perroncito, avian influenza viruses continued to infect birds in Northern Italy. There was another relatively large outbreak of disease reported in Italy 1901 (6) which spread to Austria, Germany, France and Belgium. These infections caused the classical clinical signs of a highly pathogenic avian influenza (HPAI) virus including:

drowsiness and closed eyes, little or no food consumption, foaming mouths, drooping heads, and high fever (7). Death due to HPAI can occur as little as 24 hours post-infection. Interestingly, the spread of the 1901 virus was aided when the 1901 "Brunswick Poultry Show" was closed due to the appearance of clinical signs of influenza infection. Merchants were told to take their birds home, which helped spread the once centralized virus to many regions of Germany and Belgium. After this point, HPAI viruses became relatively common in Central Europe until around 1930 (8). The first reported cases of HPAI in America occurred in 1924 and affected live-bird markets in New York City and New Jersey (9). A similar outbreak occurred in a small number of poultry flocks in New Jersey in 1929 (8). Until the middle of the 20th century, all examples of HPAI in poultry were viruses of the H7 subtype. However, in 1959 and 1961 HPAI viruses were isolated in Scotland (10) and South Africa (11), respectively, that were of the H5 subtype. In the 1970's research was performed to determine the source of avian influenza virus. The results of this research showed that a majority of influenza virus isolates were found in *Anseriformes*, consisting of geese, swans, and ducks, and *Charadriiformes*, an order consisting of terns and gulls (12). Since this time, there have been sporadic outbreaks of HPAI throughout the world. In 1983 there was an outbreak of HPAI H5 disease in Pennsylvania (13). There have also been several outbreaks of HPAI H7 viruses in Pakistan, Italy, Chile, The Netherlands and Canada in the 1990's and 2000's (8). In late 2003 there were reports of a HPAI H5N2 virus in South East Asia, an area of the world in which H5 subtype influenza A viruses had become endemic, that spread throughout that region (14). This virus has since become endemic in South East Asia and has led to infections throughout Asia, Africa and Europe. This particular H5N1 virus is particularly troubling because it has the ability to infect and cause death in humans (15).

Influenza A virus has been the known causative agent of five global human pandemics in the last one hundred years. The "Spanish Influenza" of 1918 was an H1N1 virus that caused a high mortality with about 25 million victims worldwide. The clinical picture first presented as a mild respiratory disease in swine workers that started in America. The virus was spread to Europe by American soldiers sent to fight in World War I. However, at some point the virus became very pathogenic in humans, either through a mutation event or recombination with another influenza virus, and caused a severe, systemic disease that likely induced a cytokine storm in those infected (16). Because this cytokine storm, essentially an overreaction to the virus by the host immune system, is suspected to have been the main cause of death the Spanish flu caused high mortality rates in young, healthy people as opposed to the elderly and immunocompromised individuals that is typical for influenza A virus infection (17). The "Asian Influenza" of 1957 was an H2N2 virus that emerged in China and was a reassortant between a human trophic H2N2 virus and the hemagglutinin and neuraminidase genes from an avian H2N2 virus (18). The virus quickly spread to Japan, the United States, and Great Britain eventually killing around 1 million people globally. The "Hong Kong Influenza" of 1968 was an H3N2 virus that spread globally and killed over 30,000 people in the U.S. alone. This particular virus is thought to be the result of recombination between the H5N2 Asian influenza A virus and an H3N2 strain from poultry (19). The "Russian Influenza" of 1977 was believed to be the second coming of the H1N1 1918 pandemic virus. Interestingly, this virus only killed younger age groups due to their lack of antibodies against the H1N1 virus. This virus did not originate in Russia, as the politically charged name implies, but actually emerged from northern China (20). The 2009 outbreak of H1N1 "Swine flu" resulted in a pandemic threat to the world (21). However, the death toll of this disease was not nearly as staggering as previous H1 viruses,

suggesting the virus was not as virulent. The outbreak, however, did allow the Centers for Disease Control to develop protocols for responding to future pandemic flu strains (22).

Influenza viruses are named according to a systematic nomenclature system. Taking an example of the virus A/Turkey/Wisconsin/05/1992 (H9N2) it is possible to analyze the nomenclature system. The first section of the name indicates whether the virus is influenza type A, B, or C. The second section indicates the host in which the virus was isolated, such as a turkey. If there is no species given in the name, it is assumed that species from which the virus was isolated was a human. The next section gives the location of virus isolation (Wisconsin), then a reference number for this specific virus (05), the year in which the virus was isolated (1992) and the subtype of the virus hemagglutinin (HA) and neuraminidase (NA) proteins (H9N2) (21).

Viral Genome

The genome of the influenza A virus consists of eight single-stranded, negative- sense RNA segments that encode for 11 viral proteins (23). These proteins are the polymerase basic 1 (PB1), the polymerase basic 2 (PB2), the polymerase acidic (PA), the hemagglutinin (HA), the nucleoprotein (NP), the neuraminidase (NA), the matrix 1 (M1), the matrix 2 (M2), the nonstructural (NS), and the nuclear export (NEP) proteins (3). The length of the RNA segments differ in size and can be sorted in descending size in the following order: PB2, PB1, PA, HA, NP, NA, M, NS . Each viral RNA (vRNA) segment is associated with a complex of viral proteins consisting of PB1, PB2, PA, and the NP to form the viral ribonucleoprotein (RNP) complex which is the minimal replication unit of the virus (24). Each genome segment contains, at the extreme ends, 5’ and 3’noncoding regions (NCR), but the length of these noncoding

sequences varies between each of the segments. These NCR's contain sequence elements which are necessary for incorporation of each segment into maturing viral particles. It has been proposed that the 5' and 3' NCR form secondary structures which are also called "panhandle" structures and that these structures might act as a promoter regions responsible for the transcription and replication of the viral RNA (25). Futhermore it needs to be mentioned that the extreme ends of each of the vRNA contains the exact same nucleotides, at the 5'-end and at the 3'-end (26). These sequences are thought to be responsible for packing since they are influenza A-virus specific (27)

The PB2 and PB1 segments are the longest segments with a length of 2344 nucleotides (28). Viral RNA segment 1 encodes for the polymerase basic 2 (PB2) protein. Segment 2 encodes the PB1 protein as well as the recently discovered PB1-F2 protein (29). The PB1 protein is encoded by the +0 ORF, while the PB1-F2 is encoded by a frameshift of +1 and is usually around 270 nucleotides in length. This PB1-F2 coding region has been found to be conserved in a high number of influenza A virus isolates (29).

The third viral segment encodes the polymerase acidic (PA) protein and is ~2.2 kb in length (28). The fourth segment encodes the hemagglutinin (HA) protein and is over 1.7 kb in length . Segment 5 encodes the nucleoprotein (NP) and is over 1.5 kb in length . Segment 6 encodes the neuraminidase (NA) protein and is over 1.4 kb in length (30).

Segment 7 and segment 8 both encode two proteins each and are the shortest segments contained in the virus. Segment 7 consists of more than 1000 base pairs and encodes the matrix (M1) protein and the M2 protein. The M2 protein is formed from a +1 frameshift in the vRNA and the appropriate mRNA is the result of a post-transcriptional splicing of an mRNA transcribed from the viral M segment (31). The M1 and M2 proteins contain the same amino

acids at the N-terminus. Segment 8 is around 890 bases in length and encodes for the only nonstructural protein (NS1) and the nucleoexport protein (NEP), the former NS2. The NEP protein is translated from a post-transcriptional spliced mRNA of the NS vRNA. (32). As has been described for the M1 and M2 proteins, the NS1 and NEP contain the same amino acids at the N-terminus (33).

Virion Structure

Influenza viruses are members of the family *Orthomyxoviridae*, a virus family characterized by a segmented negative-sense oriented RNA genome. The virus particle contains an envelope made of virus proteins and the cellular membrane. The influenza virus envelope is derived from the host cell during virus budding at the cellular membrane. Influenza viruses are pleomorphic, showing both spherical and filamentous, or long rod-like structures (34). The virion has 3 surface proteins. The hemagglutinin (HA) is a surface glycoprotein found as a trimer which has been proposed to associate, via the C-terminal located cytoplasmatic tail of the HA protein, with the matrix protein M1 protein inside the virion (35). The neuraminidase (NA) protein is also a surface glycoprotein formed as a homotetramer, with an interior tail that has been proposed to associate with the M1 protein within the virion (35), but both the HA and NA's interaction with M1 has been difficult to prove. The third protein which is a part of the viral envelope is the second matrix protein M2 protein which forms an ion channel connecting the outside of the virion to the inside as a homopolymer. The molecule ratio of the trimer HA to the tetramer NA is approximately 4:1 and both proteins extend from the viral envelop at around 12 nm (36). Inside the virion, the ribonuclear protein (RNP) complex is contained within a layer of M1 protein. The RNP complex consists of viral RNA (vRNA), nucleoprotein (NP), and the

polymerase complex made of the largest viral proteins PA, PB1 and PB2. These RNP structures have been shown, through electron cryo-microscopy, to be localized in the viral particle as 8 distinct RNP complexes arranged in a circular fashion (37).

It has been shown that the M1 protein is mainly responsible for the particular morphology that an influenza virion possesses. If a virus that exhibits spherical morphology is engineered with an M1 gene from a virus that exhibits filamentous morphology, the spherical virus will exhibit filamentous morphology (38). Interestingly, field strains that exhibit filamentous morphology tend to revert to spherical morphology after passaging in cell culture in laboratory settings. M1 has been shown to be the primary factor for determining viral morphology, but is not the sole factor. NA have also been shown to have some effect on virion morphology, as well as host cellular factors. These factors are not well understood, but the amount of actin in cells has been shown to have an effect on the virus morphology (39).

Viral Proteins

The 8 segments of the influenza A genome encode for 11 proteins. These proteins include the surface glycoproteins HA and NA, the ion channel M2, the structural protein M1, the proteins which are part of the ribonucleoprotein (RNP) complex (PB1, PB2, PA, and NP), as well as the non-structural proteins PB2-F1 and NS1. The NS 2 protein was thought for a long time to be a nonstructural protein but data reveal that this protein was part of the virus particle (40). Due to its function it has been named the nuclear export protein (NEP) (41). The precise structure and functions of these proteins is still not completely understood, especially in the case of the non-structural proteins.

The first three RNA segments encode for component of the viral polymerase. The viral polymerase is composed of one unit each of PB1, PB2, and PA. The polymerase is part of a viral RNP complex and is always associated with viral RNA (vRNA). The viral polymerase is an RNA-dependent RNA polymerase and is formed by PB1's N-terminus interaction with PA and PB1's C-terminus interaction with PB2 (42). The polymerase complex binds to promoters found on the vRNA segment's NCRs (43).

PB2 has been shown to contain two binding sites for NP and at least one for PB1. PB2 also has a function in inhibiting expression depending on the concentration of PB2, PB1, and NP(44). There is also some evidence that PB2 plays a small role in host tropism for influenza viruses (45).

The polymerase basic protein 1 (PB1) is responsible for polymerizing nucleotides during RNA transcription and gene replication and thus is the true RNA-dependent RNA polymerase. PB1 is also crucial in the viral polymerase's binding to vRNA or cRNA in order to initiate transcription or gene replication, respectively (46). The PB1 protein has been shown to include motifs that are conserved among RNA polymerases of many species (47). PB1 binds specifically to the terminal ends of both vRNA and viral cRNA and is necessary for elongation of RNA polymers (48).

The polymerase acidic (PA) protein is the third subunit of the viral RNA polymerase. Although little is known about the function of PA, it has been shown, by using PA mutant viruses, to be involved in both replication of the viral genome and transcription of the genes (49). The N-terminus region of the protein has been shown to have proteolytic activity, but it is currently unclear what role this activity plays in infection. The N-terminus also shows nuclear translocation activity for the protein. The C-terminus of the PA contains the PB1 binding

domains making the PA protein a necessary component of the vRNP complex (50). Recently it has been shown that PA is the responsible protein for cap snatching from cellular pre-mRNAs , a process which is necessary to form viral mRNA (51).

The hemagglutinin (HA) protein is a key antigenic protein for influenza viruses regardless of which genus. It is a surface glycoprotein whose main function is binding to host cell-surface receptors, entry of the virus into the cell, and fusion of the viral envelope with the cellular endosome to enable release of vRNP into the cytoplasm. There are currently 16 known subtypes of influenza HA (3). HA determines host cell tropism and is a key target for vaccination techniques, as it contains virus-neutralizing antibodies inducing epitopes (52). The HA exists as a homotrimer and is the most abundant protein in the viral envelope. Before binding to the host cell and internalization, the HA protein conforms to a highly compact morphology referred to as HA0 (53) The HA0 protein is made up of two distinct subunits, the HA1 and HA2. Before viral attachment, discussed later, the HA0 protein must be cleaved into the HA1 and HA2 subunits by cellular proteases for successful attachment and entry of the virus into the host cell. Lowering of the pH in the host cell endosome leads to conformational change of the HA1/HA2 molecule. During that change the structure of the HA1 changes in a way that the N-terminal domain of the HA2 protein, also called the fusion peptide, becomes accessible to fuse with the cellular membrane of the endosome. This domain allows the virus to fuse its envelope with the cellular endosome (54,55). The HA1 subunit contains a globular head that is important for viral attachment and entry into host cells (56). The HA1 subunit is also the most common target, among all influenza proteins, for virus neutralizing antibodies (57).

HA's two main roles are viral attachment and entry into host cells as well as endosome-membrane and viral-envelope fusion. The HA1 globular head contains sites that bind to host cell

sialic acid receptors. It has been shown that amino acid 226 is important in viral attachment and host cell tropism. If 226 is glutamine, the HA preferentially binds to sialic acids bound to a vicinal galactose on epithelial cell surfaces via an α 2,3-linkage. However, a leucine in the same position binds preferentially to α 2,6-linked sialic acids (58). Avian influenza viruses tend to preferentially bind to α 2,3 linkages while human isolates preferentially bind to α2,6 linkages. It is proposed that this is due to the different distribution patterns of these receptors among the different orders and/or species of animals. The membrane fusion domain is found on the N-terminus of the HA2 polypeptide. The low pH of the endosome, which is caused by the actions of M2 protein discussed later, induces a conformational change in the HA1/HA2 protein, exposing the HA2 fusion domain to the endosomal membrane. The fusion peptide forms very close and anti-parallel to the membrane domain. This brings the endosomal membrane close to contact with the viral envelope. Multiple HA2 fusion activities can lead to endosome-envelope fusion and pore forming activity that allows the viral RNP to be released into the cytoplasm (54).

The neuraminidase protein (NA) is the second major antigenic, surface glycoprotein expressed by the influenza A virus. However, unlike HA, antibodies against NA have not been shown to be neutralizing or protective in animals. There are nine known subtypes of NA. NA exists as a homotetramer and contains a hydrophobic region at the N-terminus. This hydrophobic region anchors the NA into the viral envelope (59). NA's major function is the cleavage of sialic acids on the host cell surface, specifically the α-ketosidic links between the terminal Neu5Ac and nearby saccharides. This is very important for exit of the virion from the cell (59). Without the cleavage of these sialic acid residues, the HA from the budding particle would bind receptors on the cell and prevent viral escape. NA has also been suggested to assist in viral entry, but the method of this activity is not well understood (60).

The third surface protein is the matrix protein 2 (M2). M2's main purpose is to act as an ion channel for the influx of H+ ions into the virion (61). This releases the vRNPs from their associations with M1 allowing for them to be transported to the nucleus (62), as discussed later. M2 exists as a homotetramer on the envelope surface. When the four units come together, they form a pore. This pore has been shown to be selective for H+ ions. M2 allows H+ ions from the low pH endosome to enter the virion, where they interact with M1 releasing the RNP complex after fusion (61). M2 is also important in preventing premature conformational change in HA during HA processing in the Golgi and during viral assembly (63). M2 is a highly conserved protein through all subtypes of influenza and there is ongoing research to create a vaccine that exploits this fact (64).

The matrix 1 protein (M1) is a key structural protein. M1 is the most abundant protein in the virion and is located within the viral envelope. M1 is the major determinant of viral morphology and is very important in assembly and budding of the virus (65). M1 interacts with the internal residues of the HA and NA protein and is important in bridging the outside and inside of the cell. M1 also interacts with the viral RNP complex as well as the viral envelope (66). Because M1 interacts with so many viral proteins, it is important in recruitment of viral proteins for assembly, and since it interacts with the membrane it is important for budding as well. M1 accumulates at the cell membrane, into which the glycoproteins have inserted, and recruits the RNP complexes and facilitating virion formation (66).

The nucleoprotein (NP) is the only non-polymerase protein constituent of the viral RNP complex. NP's major functions are coating the vRNA and trafficking of the vRNP complex to the nucleus. Although all of the RNP proteins contain nuclear localization signals (NLS), the only protein that is necessary for trafficking of vRNA to the nucleus is NP (67). The strongest

NLS on the NP protein is located at the N-terminal region but there is another, weaker signal towards the middle of the amino acid sequence as well as another proposed signal at the C-terminus (68). The C-terminus is also important in the binding of NP to two neighboring NP molecules to form a coating around the RNA polymer (69). Because NP is an internal protein, it is not subject to much immune pressure and in addition it is highly conserved amongst influenza A subtypes but distinguishable from the NP of influenza B and C. This lends NP to be a good target for potential diagnostic testing (70).

The PB1-F2 is a small, non-structural protein that results from a +1 frameshift of the PB1 RNA segment. The PB1-F2 is a small protein usually consisting of 90 amino acids, but sometimes consisting of fewer (71). PB1-F2 was recently discovered in the A/Puerto Rico/8/34 (H1N1) virus, otherwise known as PR8, but further analysis of other isolates revealed that most influenza isolates encode the PB1-F2 frame shift (72). The protein has been shown to possess pro-apoptotic activity and also plays a role in interfering with host cell innate immune responses. PB1-F2 has been shown to localize at mitochondria in the host cell where it interrupts the membrane integrity and can lead to rupture of mitochondria (72).

The non-structural protein 1 (NS1) is a small protein that is important in down-regulating the cellular innate response to viral infection and the shutdown of the host cell protein machinery. NS1 exists as a homodimer and can bind to dsRNA (73). This is important because cellular recognition of dsRNA will lead to a type I interferon response. Viruses engineered to lack the NS1 gene have been shown to be highly susceptible to host interferon responses, reinforcing NS1's role in interrupting interferon response (74). NS1 also interacts with poly(A)-binding protein II (PABII) preventing the polyadenylation and thus the translation of host cell mRNAs. NS1 can also shut down host pre-mRNA splicing by interacting with cleavage and

polyadenylation specificity factor (75). The shutdown of the cell interferon response gives an advantage to the virus which is intensified by the shutdown of the host cells ability to produce functional mRNAs.

The nuclear export protein (NEP), formerly known as NS2, is important in exporting the RNP complex out of the nucleus. It interacts with Crm1, a cellular export receptor (76). It also interacts with M1, and is theorized to form a complex with M1 which enables the entire RNP/M1 complex to be exported from the nucleus by the Crm1/exportin1 pathway.

Viral Replication

Influenza A viral replication is a process that is comparably well, though not completely, understood. The viral replication cycle occurs in many distinct steps. These steps include: attachment and adsorption, endocytosis, fusion and uncoating of the virus, transport of vRNP to the cell nucleus, transcription and translation of viral genes, replication of the viral genome, post-translational processing of viral proteins and transport to the cell membrane, packaging, and finally budding of the functional virion. These events occur within different compartments of the cells and the virus depends on both cellular machinery and constituent signals to translocate and perform these functions.

The influenza A viral replication cycle begins with virus attachment to target host cells. The HA protein binds to sialic acid receptors on the cell surface (77). Viruses that show a preference for infecting avian species preferentially bind to sialic acids containing an α-2,3 linkage between the N-acetylneuraminic acid and the galactose sugar contained at the terminal end of the receptor. In influenza A viruses infecting humans, the HA preferentially binds to sialic acids containing an α-2,6 linkage (77). However, after infection of an individual, it has

been shown that a virus can mutate and change its preferred receptor affinity. This can be achieved by a single amino acid mutation and can allow for a virus to adapt to its host and allow for more efficient infection of neighboring cells (78).

After the virion has attached to a sialic acid receptor it undergoes the process of entry into the cell. This process involves the virus being taken into a cellular endosome. Viral entry requires endosomic activity because the HA protein requires a low pH to initiate the fusion process that will be discussed later (79). The method by which influenza viruses induce the cell to perform endocytosis on the virion is not well understood, but it is most likely due to stimulation of the appropriate sialic acid containing receptor molecule. Once stimulated, these receptors call for the virion to be pulled into the cell along with a portion of the cellular membrane (80).

Once the virion is inside the endosome and transported into the cytoplasm, the physiological cellular process of lowering pH in the endosome takes place. This lowering of the pH causes a conformational change of the viral HA protein. The HA protein exists naturally as a homotrimer with each subunit of this trimer containing and HA1 and HA2 protein subunit. Prior to entry into the cell, these proteins are cleaved from their joined state (HA0) into their individual HA1 and HA2 subunits. This cleavage is performed by cellular proteases that are either extracellular or intracellular (81). The HA2 subunit is anchored to the viral membrane by its hydrophobic C-terminal domain and the HA1 subunit is bound to the HA2 subunit via a hydrogen-bridge provided by the sulfur-containing amino acid cytsteine (82). Once in the presence of the low pH of the endosome, the N-terminal of the HA2 subunit located hydrophobic fusion peptide is exposed to the endosomal membrane. Once several HA fusion peptides are brought together at the endosomal membrane, a pore forms allowing the viral RNP material to be

released into the cytoplasm of the cell (83). In parallel, the movement of H+ ions into the viral particle is facilitated by the ion pore forming viral M2 protein. This lowers the pH within the virion, allowing the RNP complexes to dissociate from the M1 protein and be released from the virion (84). Once released from their interaction with the M1 protein the RNP can move through the pore formed by the HA aggregates and into the cytoplasm and are moved to the nucleus using inherent nuclear localization signals located on the proteins of the RNPs. The process from attachment to uncoating takes as little as twenty five minutes (85).

Once the vRNP complex is translocated to the nucleus, mRNA synthesis is initiated. The synthesis of viral mRNA is dependent on the viral RNA polymerases, but viral mRNA must receive a 5' cap from cellular sources. Cap-snatching is facilitated, in concert, by the PB1 and PB2 proteins. The PB2 protein binds cellular pre-mRNA caps and the PB1 protein cleaves them so they can be used for the mRNA during synthesis on the vRNA (86). Once supplied with a cap and primer for elongation, the viral polymerase complex is able to perform transcription of the viral RNA sequences producing viral mRNA. This elongation continues until the PB1 subunit of the polymerase complex encounters a region of repeated uridine bases, which is the signal for polyadenylation of the mRNA (87). Once this signal is recognized, the polymerase is blocked from transcribing the message further by means of loop structures in the RNA template. This causes the polymerase to stutter on the uridine residue signal and produce a poly-A tail necessary for transport out of the nucleus and translation of the mRNA in the cytoplasm (88).

The method by which the vRNA is copied into complementary RNA (cRNA) for purposes of gene replication is different from the method of producing viral mRNAs. cRNA is produced without a capped primer and copies the full length of the viral gene, necessitating that there be no stuttering at the polyadenylation signal. The method by which the polymerase

complex achieves this is not well understood, but it is proposed that soluble NP has a function in switching from mRNA production to cRNA production (89). This has been theorized because NP must be synthesized before cRNA production begins and viral NP is not sufficient for cRNA production (90). Once viral cRNA has been produced, the viral polymerase complex can use these templates to make copies of vRNA.

Once new vRNA is synthesized, it associates with imported NP and the viral RNA polymerase to form vRNP complexes. It has been shown that the M1 protein interacts with the NP on the vRNP complex and that this interaction is necessary for transport of the new vRNPs from the nucleus into the cytoplasm. It has been proposed that the M1 protein is necessary to disrupt the interaction of the vRNP from the nuclear matrix components (91). Once the vRNP is assembled in the nucleus the viral export protein NEP (former NS2 protein) interacts with cellular nuclear export machinery to facilitate the movement of vRNP into the cytoplasm (92).

Once synthesized on ribosomes, the HA, NA, and M2 proteins are processed in the endoplasmic reticulum (ER). In the ER, the HA and NA are glycosylated and all three of the membrane associated proteins are formed into their respective multimers (93,94). Proceeding their processing and assembly in the ER, the membrane proteins are transported to the Golgi apparatus via the trans-Golgi network for further processing. In the Golgi, the HA and M2 have cysteine residues palmitoylated to help facilitate placement in the cellular membrane (95,96). Viral assembly occurs at the apical membrane of the cell. This can be a reason that influenza infections tend to be more local instead of systemic in nature (97). The membrane proteins contain signals in their transmembrane domains that cause them to be transported to the apical membrane of the cell (98, 99). They are carried to the membrane on lipid rafts (98).

Once the membrane proteins are at the surface of the cell, packaging and budding of the new virion can begin. Little is known about how vRNAs are brought together to form a whole virion. It has been proposed that each RNA segment contains a packaging signal and that, when a full complement of these segments is included in a virion, budding is initiated. This has been proposed because of analysis of the NCR of the RNA segments. When a wild-type NCR of the virus was added to a reporter sequence, that sequence is more likely to be incorporated into the virion (100,101). However, these packaging sequences are not virion specific and the replication of two viruses in one cell can lead to the mixing of segments among the viruses. This can lead to the egress of recombinant viruses that can potentially be more infectious than the two original viruses (102).

The M1 protein that is associated with the vRNP also associates with the membrane proteins on the cellular surface. Once enough M1 has accumulated near the cell surface, the cell membrane curves outwards and continues until the viral components are encapsulated in the membrane. This process requires M1 to be present (103). After budding, the virion must be released from the cell due to HA protein's interactions with cellular surface sialic acids. This function is performed by the NA protein. The NA cleaves sialic acids from cellular and viral molecules. It has been shown that without NA activity, influenza viruses will aggregate at the cell surface and with other virions (104).

Diagnostic Techniques

Because of the variety of clinical signs that influenza infected birds can exhibit, and the fact that various agents cause diseases resulting in similar clinical pictures, viral diagnostics are a key factor in surveillance and control of avian influenza worldwide. Because of this, many

diagnostic tests have been developed and are being developed to identify and classify influenza A and B virus infection in humans and mainly influenza A virus infections in animal species. In poultry, methods for diagnostic evaluation include virus isolation in the embryonated chicken egg, hemagglutination test (HA test), agar gel immunodiffusion test (AGID), hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, reverse transcriptase polymerase chain reaction (RT-PCR), and the enzyme-linked immunosorbence assay (ELISA). These tests were designed to detect the presence of infectious influenza virus, or antibodies to influenza virus, and to indicate the antigenic subtype of the influenza A virus. RT-PCR was initially designed to detect influenza virus RNA and has been developed to a set of tests where the presence of influenza A virus genome is initially detected and, in parallel, a subtyping of important HA subtypes for poultry(H5 and H7) are performed.

Virus isolation (VI) is the first step in determining the presence of influenza virus in a sample and is useful in creating a virus stock to be further analyzed and typed. The most common medium for VI tests is the embryonated chicken egg from a flock which does not have any influenza A antibodies (105). Although it is possible to perform VI in cell lines, eggs are more sensitive and the most accommodating to all influenza A types in birds which is an important factor when the subtype and specificity of a sample is unknown. VI in eggs can be a time-consuming process, especially if the sample in question must be passaged several times in eggs before becoming detectable by means of other diagnostic assays and has been shown to be less sensitive than other, more recently developed molecular diagnostic methods (106). Once a sample has been successfully passaged in eggs, the virus isolated from the eggs can be used in further diagnostic testing.

The HA assay is a quick, cheap, and relatively simple way to test a sample for presence of a hemagglutinating agent. It needs to be mentioned that other agents, including bacteria and viruses, besides influenza A viruses are able to agglutinate red blood cells. The HA test is based on the principle that the influenza A virus encoded surface protein hemagglutinin can bind to red blood cells causing agglutination which is detected by the formation of aggregates which are unable to form a dot-like spot at the bottom of the U – or Y shaped well. A sample is serially diluted and mixed with avian red blood cells and hemagglutination becomes vissible. Although this method is very sensitive for influenza A virus, it has somewhat low specificity because of the ability of other avian pathogens to induce hemagglutination in avian erythrocytes, especially Newcastle Disease Virus and several bacterial strains (107). Because of the low specificity of the test, further diagnostic tests for typing are recommended for samples that have been tested positive in the HA test.

The gold standard for both antibody and antigen detecting in influenza A virus is the agar gel immunodiffusion assay (AGID). AGID testing can be used to indicate the presence of viral antigen belonging to influenza A virus in a sample, the presence of anti-influenza antibodies in a serum sample, and can be used to type either the antigen or antibodies in a clinical sample. The basis for this testing is the specific antigenicity of the NP protein of influenza A virus which can be used to differentiate between influenza A, B and C. Besides NP also the M1 protein can be used. Wells are cut into an agarose gel substrate in a circle around a central well. In the central well, either an unknown or reference influenza antigen(A, B, or C) is placed and allowed to diffuse into the surrounding gel. In the outer circle, either reference sera or unknown sera are allowed to react to the antigen which results in a visible, white precipitate. The AGID test takes 24-48 hours and requires some experience to interpret results and is therefore relatively

expensive. However, it is a very reliable way to determine the presence and/or type of influenza virus or antibodies in an unknown sample (108).

A method commonly used to type viruses or determine the amount of antibodies in a serum sample is the HI assay . The HI test involves incubating viral antigen with a serially diluted antibody solution and measuring at what dilution the antibody solution is still able to inhibit the hemagglutinating activity of the viral antigen. The antibodies' inhibition of hemagglutinin activity is influenza A virus subtype specific, and thus the test can be used to determine the presence of antibodies for a specific influaenza A virus subtype in a serum sample or the subtype of an unknown antigen when used against a serum sample which is directed against a known HA subtype. The HI test can be carried out in less than 2 hours and generally shows a high specificity. It needs to be mentioned that the specificity can be compromised if there has been a significant antigenic drift in the newly isolated influenza A virus virus and the appropriate reference serum (109). The neuraminidase inhibition assay (NI) is an assay using a similar method. Antibodies against the NA protein block the intrinsic enzymatic activity of the protein to cleave a substrate and produce a color change in the appropriate assay. The inhibition of the color change is measured against a control, thus allowing for the percent inhibition of the NA protein by a specified sera to be quantified. The NI test is mainly used as a method of typing viruses and is not often used to determine the presence or absence of antigen or antibodies in a test sample due to its high demand on enzymatic reactions and the fact that it is mainly performed in specialized laboratories (110,111).

A very accurate and relatively quick method for typing influenza viruses is by using reverse-transcriptase polymerase chain reaction (RT-PCR). Using primers specific for influenza A viruses, the viral RNA genes can be amplified to a point that is detectable and candidate for

sequencing (112). This method is very specific and very sensitive, requiring as little as 5 viral RNA copies for detection (113). Using RT-PCR for isolation of viral genes and then sequencing these genes allows for fast and accurate subtyping of the influenza viruses. This is a very reliable, quick diagnostic method, but it is expensive and cannot be carried out in the field due to its high demand on technical equipment. Therefore, it remains an important and very well used laboratory tool.

ELISA systems are useful for determining serum antibody levels against influenza virus. These tests are relatively inexpensive, fast, and are capable of being used in the field. These factors make ELISAs good candidates for broad diagnostic activities. ELISAs have been shown to have higher sensitivity than AGID but also pose problems due to the test's low specificity and high false-positive rate (114). However, commercial ELISA kits are specific for only a single species, which can lead to high costs to cover multiple species. In order to get around this inconvenience, competitive ELISAs (cELISA) have been developed and are under development (114). cELISAs use a monoclonal antibody directed against an influenza virus protein as a detection method. When a serum containing influenza antibodies from any species is used as primary in an ELISA plate, these antibodies inhibit binding of the monoclonal which is added to the ELISA plate after the serum in question has been added. This inhibition can be measured and used to determine if a given is positive for influenza antibodies, regardless of species. There are several tests on the market which use the NP of influenza A virus as target to screen serum samples for the presence of NP antibodies. These ELISA are available as indirect and also as cELISA and have become an important tool for screening the tremendous amount of chicken sera, to test for the absence of influenza A virus antibodies, at processing plants and other occasions where such infection is assumed.

Vaccine Technology

Influenza outbreaks are a major concern to the vitality of the poultry industry and also pose a risk to human populations, especially those humans that come in contact with infected poultry. This has been shown to be true in the recent outbreaks of highly pathogenic avian influenza (HPAI) H5N1 in Southeast Asia where birds infected with HPAI H5N1 were able to transmit the virus to people who worked closely with infected birds. In several countries where outbreaks of HPAI H5N1 in poultry occurred, infections of birds of prey, ferrets, felids, and humans with fatal outcomes were also reported (115). The major health concerns that this raises for both humans and poultry, especially considering influenza A virus' ability to mutate, leading to an antigenic drift, or the exchange of antigenically important HA or NA, leading to a antigenic shift. These mechanisms of viral evolution requires a fast, effective means for preventing outbreaks and stopping spread of virus from its location of incidence (116). In the past, the most common method for preventing spread of avian influenza virus was to isolate infected birds. It was noticed that some birds were not killed by the virus, and were subsequently protected from reinfection. Protection from reinfection likely means that there were virus-neutralizing antibodies present in the serum of infected animals (117). If these protective antibodies can be produced without an infection of the dangerous virus, there is a chance to lessen the risk of poultry infection. Vaccination is an effective and long established method for inducing protective immunity in animals and humans. There have been two basic types of vaccines developed against influenza virus infection: attenuated viruses, which eventually can be used as live vaccines, and vaccines based on inactivated viruses, and more recently the use of

recombinant vaccines where the protection inducing antigen was genetically inserted in the vector vaccine.

Influenza virus vaccine development faces many obstacles. The most important obstacle is the antigenic diversity of influenza A viruses. The vast majority of influenza A virus vaccines focused on producing antibodies mainly against the HA protein. If enough antigenic drift has occurred within an HA subtype, antibodies against homologous subtypes may not be sufficiently protective, even if the HA subtype is the same but there has been sufficient mutations within the protein (118). Due to the influenza virus' ability to mutate the antigenically important protein (antigenic drift) and exchange whole RNA segments encoding for the antigenic important antigen (antigenic shift), a substantial obstacle to effective vaccination is faced. Another problem with production of influenza A virus vaccines is related to the technical difficulties of working with HPAI. HPAI must be worked with under high biosafety conditions, a fact which is expensive and limiting in both time and resources. This characteristic limits the production and alternatives have been developed. These methods are based on the closest antigenic fit the circulating viruses. This limits the ability to respond quickly to outbreaks of novel influenza A virus subtypes and makes vaccination, for extensive use in the poultry industry, expensive. Currently, avian influenza vaccines are given intramuscularly which is an expensive and labor-intensive process (119,120). Another problem that vaccine developers must get around is differentiating infected from vaccinated animals, or DIVA. Every vaccine produced must include a method by which a diagnostic assay can discriminate whether a bird has been infected with an influenza virus or has only been exposed to the vaccine. A common method of ensuring DIVA in vaccines is to engineer a vaccine so that it has a very distinct set of genes or, if using a subunit vaccine, is missing a particular protein necessary for whole virus replication (121).

The most popular avian influenza virus vaccine for poultry is the inactivated vaccine based of the whole virus. These vaccines are composed of LPAI that has been inactivated by chemical detergents such as formalin, beta-propiolactone, or ethyleneimine (122-124). These vaccines must be administered subcutaneously or intramuscularly and require adjuvants to be effective. These vaccines also require a relatively high amount of antigen to protect birds from clinical disease and death. The antigen for these vaccines is produced in embryonated chicken eggs (122). This is a time-consuming process that has been estimated by the CDC to take 6 months to produce enough vaccine to respond to an epidemic situation in humans, and would take even longer to make enough effective doses for the poultry industry. Because of this, there has been effort recently to produce these viruses by using HA and NA from a circulating flu strain and the other 6 RNA segments from a fast-growing LPAI strain (125). The development and use of reverse genetic systems to produce flock specific inactivated virus vaccines has become a more common practice in the poultry industry (121). These vaccines can also be engineered so they can be used for DIVA analysis.

Live-attenuated vaccines are not used in poultry for fear of the virus becoming more pathogenic once introduced to the flock. However, the vaccine is produced for use in humans, but must use a virus that is cold-adapted and sensitive to temperature (126). Live attenuated vaccines gain an advantage because they can be administered via a nasal spray in humans, like the FDA approved vaccine FLUMIST, and induce a stronger immune response than inactivated virus vaccines (127). Previously, attenuated LPAI viruses were created by passaging the viruses in cells or eggs at a low temperature, thus cold-adapting them. Currently, most of the live-attenuated vaccines are produced by introducing HA and NA genes from circulating viruses to the backbone of an influenza virus that has already been cold-adapted using reverse genetics

(128). There has also been success with generating recombinant adenoviruses, herpesviruses, and paramyxoviruses that express influenza HA and NA proteins in experimental settings (129-131). So far one recombinant fowl poxvirus encoding for an H5 protein has been licensed in US (132). This vaccine uses a fowl poxvirus backbone to express the H5 protein of influenza. The poxvirus can replicate in the bird, but produces no clinical signs and is cleared in healthy birds, and in the course of infection exposes the host to target influenza proteins. This vaccine also has DIVA capabilities. Live-attenuated vaccines are useful because they not only produce a humoral response, but are also effective in eliciting a cellular response. This leads to a better level and percentage of protection using smaller amounts antigen.

Subunit vaccines involve the *in vitro* production of influenza HA protein for use in vaccine production. These vaccines are usually produced via vectors containing plasmids containing the whole or partial influenza HA gene. These vectors can be bacteria, plant, or yeast cells, or most commonly viral backbones (132-135). The most popular viral vector is the baculovirus, which only infects insect cells, because of the reduced risk of contamination and the inability of baculoviruses to infect poultry or mammals (136) These vaccines are useful because they can be produced industrially and with none of the danger of producing influenza viruses in embryonated chicken eggs. However, these vaccines suffer from some of the same setbacks of the inactivated whole virus vaccines, the most important of which being the labor and expense of vaccinating poultry intramuscularly.

A new avenue of research involves using DNA vaccines. DNA vaccines are created by inserting influenza cDNA encoding for protection inducing protein(s) into plasmids containing promoters which are binding sites of the cellular DNA-dependent RNA-polymerase II. These plasmids can contain multiple HA and NA types and thus can offer protection against many

subtypes of influenza. These plasmids are taken up by cells and produce the viral proteins which are then presented on the cell surface (137). Such vaccines are effective at eliciting both a humoral and cellular immune response. However, their efficacy in chickens has been inconsistently shown, mainly due to lack of knowledge of effective promoters in chickens (138), and they are much too expensive at the current time to be taken as a serious candidate for poultry vaccination (139).

Because many of the influenza vaccines require a large amount of antigen and several immunizations to induce protective immune responses, adjuvants are very important when formulating influenza vaccines. Adjuvants are non-viral chemicals that stimulate the immune system to respond to proteins in the vaccine. The most commonly used adjuvants are composed of a mixture of oil and water. When injected intramuscularly, they irritate surrounding tissue and activate immune responses in the tissue. Vaccines that use this adjuvant have been shown to induce high antibody titers in vaccinated animals and an extended period of time in which the vaccinated target retains protection from the virus (140). Other adjuvants used in influenza vaccines include aluminum salts, liposomes, microparticles, cytokines, and bacterial proteins among others (141).

Virus-like Particles

A virus-like particle (VLP) is a collection of a viral protein or viral proteins that forms a unit that resembles the natural viral particle structure (142). VLPs contain no viral genetic material, and therefore cannot replicate within host cells. VLPs can be produced that mimic viruses from different virus families of both enveloped and non-enveloped viruses. VLPs have been generated from viruses that have single or multiple capsid proteins as well. It has been

observed that when capsid proteins, or other viral proteins being part of the outer shell of the viral particle, were expressed in cell culture, some viral proteins spontaneously form particles that were similar in morphology to the original virus (143,144). Because of the similarities between natural viruses and their VLPs, these structures induced antigen-presenting cells (APCs) and dendritic cells (DCs) to activate and present the processed proteins to lymphocytes (145,146). VLPs have been shown to not only elicit B cell response, which can be measured by the presence of antibodies, but also induce Tcell responses wherein CD4 positive and cytotoxic T lymphocytes were involved (147,148). Because of this capability, VLPs may be able to induce a more profound protection than normal subunit vaccines *in vivo*.

There are several methods by which VLPs can be produced. The most common method is to produce VLPs in heterologous expression systems. There are two main methods by which to do this. The first method is the expression of viral proteins in prokaryotic or yeast cells. This is achieved through either transformation or reverse engineering of target cells. The hepatitis B virus (HBV) vaccine is an example of a yeast expressed VLP vaccine that has been very commercially successful. The HBV vaccine was, in fact, the first commercially produced and approved VLP vaccine (149). Another method involves the infection of target cells by recombinant baculoviruses encoding the genes of interest to form the VLPs. Using insect cells to create VLPs for use in vaccines has many distinct advantages. The ability of insect cells to produce a large amount of recombinant protein and subsequentely intact VLPs means that this system might be cheap. In addition, by the current available technologies, the recombinant baculovirus can be easily manipulated. Furthermore, available technologies, e.g. 500 l to 1000 l Wave technologies, allow industrial level manufacturing (150). The use of insect cells minimizes the possibility of contamination of vaccine supplies with organisms or factors that are

harmful to mammalian or avian species (142). Baculoviruses have a very limited host range and pose no threat to humans or poultry. They also can be easily neutralized in culture (151) to prevent spread into the environment. For these reasons, the most common expression system for VLPs in technological developments is the recombinant baculovirus system. Another, very rarely used, method of production involves the infection of mammalian cells with recombinant vaccinia virus. This method is disadvantageous due to the fact that there is a greater risk of contamination with mammalian pathogens along with other factors (142). Another promising production method involves using recombinant plants to produce VLPs. Plant chloroplast or nuclear genes are stably transformed and will bud VLPs when presented with compatible viral proteins. This research is still in its early stages, but has produced some promising results (152).

There are several VLPs being developed for vaccination and a few that are already in production and being sold as vaccines. The first successful VLP based vaccine was the HBV vaccine mentioned earlier. However, the most well-known and arguably most important VLP based vaccine is the human papilloma virus (HPV) VLP vaccine. The vaccine Gardasil is composed of the L1 genes of four HPV serotypes (HPV 6, HPV 11, HPV 16, HPV 18) and is produced in yeast (153). The second available HPV VLP vaccine (Cervarix) is produced in insect cells and encodes for two serotypes, HPV 16 and HPV 18 (154). Both of these preparations were shown to be effective in preventing, or at least reducing, HPV infection of the appropriate serotypes in female humans, thus eliminating the risk of cervical cancer as well as associated cancers. Based on this success, the use of VLP-based HPV vaccines in male humans is under discussion to break infection chains. Besides these examples, there are several VLP vaccines in development for use in human as well as veterinary medicine. These include members of the *Calciviridae, Picornaviridae, Flaviviridae, Retroviridae, Paramyxoviridae,*

Orthomyxoviridae, Bunyaviridae, Burnaviridae, Reoviridae, Parvoviridae, Circoviridae, and *Polyomaviridae* virus families (142).

Influenza virus-based VLPs (Influenza VLPs) have been successfully produced and offer distinct advantages as vaccine candidates. Influenza VLPs have been produced by a number of different methods, including recombinant vaccinia virus expression in mammal cells, baculovirus expression in insect cells, and, recently, expression in plant systems among other systems. Mammalian expression systems rely on the co-expression of NA on the VLP. Without the expression of this enzymatic active protein NA, VLPs are not capable of releasing from the cell surface after budding due to the inability to cleave themselves from sialic acid residues on the cell surface. However, not all VLP preparations require the expression of the NA protein. In insect cell culture, NA is not necessary and the expression of influenza M1 protein alone is sufficient for the formation of vesicular particles that were similar to VLPs (155). Generally it has been assumed that M1 is crucial; for VLP production, with HA's and NA's importance being secondary in importance, depending on the expression system and expectations of the use of the VLP. However, there have been recorded instances of VLPs being formed without the expression of M1 protein in DNA-vector expressed systems (156). The mechanism of the budding and forming of VLPs in various expression systems is not well understood and is in need of further research.

By far the most common method for producing influenza VLPs intended for vaccine use is the recombinant baculovirus expression system. To date, VLPs have been produced via this method containing the HA, NA, M1, M2; the HA, NA, M1; or just the HA and M1 proteins (155,157,158). These particles have been shown to produce a robust immune response in mammalian hosts, leading to protection from infection in multiple species. However, the

research covering the ability of VLPs to protect poultry has been lagging. The distinction between using HA protein subunit vaccines and VLP vaccines has been shown by heat-treating influenza VLPs before delivery to mice. While the intact VLPs led to a robust immune response and protection of the mice from lethal challenge, the heat-treated VLPs were unable to provide any protection or immune response (159). These data reinforce the need for intact VLPs for proper immune response to viral VLPs.

The importance of the development of influenza VLPs for vaccine production cannot be understated. As discussed earlier, current influenza vaccine technology is relatively slow and expensive. These facts do not lend influenza vaccines as a viable option for extensive use in the poultry industry or as a suitable solution for response to novel, pandemic strains of influenza. VLPs produced in insect culture allow for vaccine developers to maneuver around many of the obstacles facing effective influenza vaccines for poultry. Insect cell expression systems can produce a large quantity of viral proteins, and thus a large amount of viral VLPs compared to other recombinant expression systems. This leads to a cheaper and faster way to produce large amounts of antigen for immunizing of animals. As stated previously, current vaccine technology demands that animals receive the influenza vaccine through injection. This is a time-consuming and labor-intensive process and thus is a limiting factor in poultry influenza A vaccination. VLPs, however, have shown efficacy through intransal delivery and preliminary efficacy through aerosol delivery in mammals. The potential for VLPs to be delivered via aerosol would greatly reduce the cost and labor needed to deliver effect influenza vaccines to poultry (160). Also, the specificity and speed with which VLPs can be produced in insect cell culture lends itself to response to the emergence of novel strains of pathogenic influenza viruses in the field. The rapid production of VLPs in cell culture when compared to the production of vaccine in embryonated

eggs can allow for a much more rapid response to pandemic influenza strains. These factors could lead to the protection of millions of animals and the equivalent amount of money associated with the production of poultry worldwide.

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CHAPTER 3

AEROSOL VACCINATION OF CHICKENS WITH BACULOVIRUS EXPRESSED VIRUS-LIKE PARTICLES INDUCED IMMUNE RESPONSE IN CHICKENS

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Abstract

Influenza A virus (IAV) vaccination of animals and humans is a powerful tool for prevention and control of infection and disease. Currently licensed vaccines are egg-based and delivered by injection which is labor intensive. As an alternative vaccine manufacturing method, baculovirus grown in insect cells cultures can produce high yields of virus-like particles (VLP) which contain viral proteins but lack genetic material, and thus are unable to replicate. VLP vaccines have been shown to be highly immunogenic after parenteral application in mice, ferrets, and humans. The aim of this study was to assess influenza VLPs, as an aerosolized vaccine (AE), as a vaccination strategy in chickens. VLPs used in this study were composed of IAV hemagglutinin and matrix protein 1. Plethysymography was used to determine the respiratory parameters for the chickens and to calibrate a controlled VLP aerosol application dose. One-day-old SPF chickens were vaccinated twice, 14 days apart, with 100 μg, 20 μg, or 5 μg of VLPs. As control, chickens were also vaccinated via intranasal (IN) instillation and intramuscular (IM) injection. Serum samples were tested for the presence of neutralizing and HI antibodies, or by indirect ELISA using baculovirus expressed H5-Vietnam protein. The VLPs induced seroconversion after IM application at any dosage. In contrast, aerolized VLPs induced a specific antibody response after AE but only when 100 μg were given. These data show for the first time that non-replicating influenza VLPs might be used for mass aerosol vaccination in chickens.

Keywords: Influenza virus, virus like particle, aerosol, mass vaccination.

Introduction

Avian influenza (AI) is caused by viruses of the family *Orthomyxoviridae* which contain a segmented, single-stranded RNA genome of negative sense orientation. *Orthomyxovirida* have been divided into the genera *Influenzaviruses A, Influenzaviruses B, Influenzaviruses C, Thogotovirus, and Isavirus* (Knipe et al., 2007). Influenza A, B, and C are divided by antigenic properties of two of their internal proteins, the matrix protein M1 and nucleoprotein (Webster et al., 1992). Influenza A subtype viruses are the only members within the family *Orthomyxoviridae* known be able to infect birds (Alexander, 2000). All influenza A viruses are classified based on their hemagglutinin (HA) and neuraminidase (NA). Sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes can be distinguished using appropriate serologic tests (Hinshaw et al., 1982; Kawaoka et al., 1990; Rohm et al., 1996; Fouchier et al., 2005) which would result in 144 different possible combinations. Since being initially describe, AI has been an economic threat to commercial poultry worldwide. Since 1997, direct avian-to-human transmission of lethal AI viruses of the H5 subtype has elevated the need to control AI beyond economic considerations. Several approaches have been used to control AI in poultry settings with the use of inactivated whole influenza virus being the oldest approach to control outbreaks of highly pathogenic avian influenza virus (HPAI). Low pathogenic avian influenza viruses, (LPAI) isolated from outbreaks in poultry or from surveillance of birds, have been used in inactivated AI vaccines. These vaccines have been a proven, low cost technology used for over 30 years (Swayne, 2009). Another experimental approach was the use of heterologous expressed H5 and H7 (Crawford et al., 1999) or H5 (Lin et al, 2008) proteins for use as a vaccine. These subunit vaccines were able to provide protection in appropriate challenge experiments in chickens. Also, the use of viral vector systems, which facilitate the expression of the protection

inducing HA antigen, in poultry was extensively analyzed and showed promising results. Here the use of recombinant viruses such as infectious laryngotracheitis virus (Lüschow et al, 2001), vaccinia virus (Poon et al, 2009), fowl pox virus (Webster et al., 1996), Newcastle disease virus (Veits et al., 2006) and also non-replicating adenovirus (Toro et al., 2008) has been investigated for poultry and protection was observed in birds which did not have any antibodies directed against the vector virus. Another approach is the administration of DNA-based vaccines which encode for one or more of the influenza virus proteins. The use of such vaccines in chickens might be limited due to the high costs for repeated administration and production of the vaccines. Nevertheless, protection in challenge experiments has been shown using an HA5-encoding (Kodihalli et al., 1997) or HA7-encoding (Jiang et al, 2010) plasmid as a vaccine candidate. In a systematic approach, Suarez and Schulz-Cherry (2000) showed that the presence of certain DNA-dependent RNA-polymerase II promoters in the plasmid influenced the expression of the target HA protein and, consequentially, improved protection in subsequent challenge experiments. Another approach was the use of AI viruses which encode for the truncated nonstructural protein 1 (NS1) in chickens. Truncation of NS1 of influenza A viruses led to viral attenuation due to an inefficient suppression of the innate immune response in the host (García-Sastre et al., 1998). Using influenza A reverse genetics, it has been shown that the truncation of the NS1 protein attenuates AI viruses in chickens and these viruses, used as live vaccines in experimental approaches, were able to induce protection from lethal challenge (Wang et al, 2008, Steel et al, 2009).

A different approach is the use of recombinant DNA technology to mimic the natural structure of the viral particle. These mimicked particles have been labeled as virus like particles (VLPs). To this end, proteins necessary to form the VLP were expressed in heterologous systems and

subsequently used as vaccines. The advantage of this system is that a structure that closely resembles that of the virus particle is formed which usually also allows for formation of antigenic structures able to induce protective immunity in the appropriate host. VLPs were first described over 50 years ago for papilloma viruses as particles which were similar to viruses but not infectious (Strauss et al., 1950). These findings indicated that viruses can form their natural virus structure without having the genomic information as part of this structure. The discovery of VLPs in cells of tumor origin led to the conclusion, that, although no infection was experimentally performed, viruses might be part of the induction of such tumors (Epstein, 1955; Oberling et al, 1957). This conclusion was supported when Thiery et al (1959) found VLPs in a chemically induced carcinoma of the uterine cervix. With the use of recombinant DNA technology, experiments were performed to express the hepatitis B surface antigen (HBsAg) in heterologous systems (yeast cells, in this case) and to determine their effectiveness for use as a vaccine (DeWilde et al, 1985). This recombinant protein could be a substitute for the prospective use of natural inactivated HBsAg obtained from human serum samples (Krugman, 1975), the use of which was approved in 1981 (Krugman, 1982a). With this development, it was assumed that the next generation of Hepatitis B vaccines would be based on HBsAg obtained by recombinant DNA technology (Krugman, 1982b). As mentioned above, his type of vaccine was already under development (DeWilde et al 1985) and recombinant HBsAg has been tested in clinical trials with promising results (Zuckermann, 1987) and was released in Belgium in 1988 (Harvengt, 1988). Driven by this success for other viral systems, attempts were made to produce VLP's based on recombinant DNA technology for the following viruses: reoviruses (Roy et al, 1992), human immunodeficiency virus 1 (Wagner et al, 1992), parvoviruses (Martínez et al, 1992), papilliomaviruses (Kirnbauer et al., 1992), and rotaviruses (Redmond et al., 1993). These VLP

vaccine candidates were mainly based on single protein expression systems which resulted in the formation of a VLP. In a more complex approach, Hobmann et al. (1994) produced VLPs in eukaryotic cells based on the glycoproteins (E1, E2) and the capsid protein of rubellavirus. This was a more complex system and opened up a new avenue for generation of VLPs for enveloped viruses. A similar approach was performed for the enveloped Hepatitis C virus using a baculovirus-based system (Baumert et al., 1998). The formation of influenza virus VLPs was already described in the 1950's (Werner and Schlesinger, 1954) and the formation of VLP of influenza viruses based on recombinant DNA technology was first described by Latham and Galarza (2001) using a recombinant baculovirus encoding for four influenza A virus proteins. These VLPs were able to provide protection after intranasal and intramuscular application in mice (Galarza et al, 2005). The successful use of similar influenza A virus VLP based vaccines in mice has been shown by several groups (Quan et al, 2007, Bright et al., 2007, Mahmood et al, 2008, D'Aoust et al., 2008). The use of influenza virus VLP antigens for use as a vaccine has been poorly investigated in poultry. Although the formation of infectious bursal disease virus VLPs has been observed following expression in baculovirus based systems (Fernández-Arias et al, 1998, Kibenge et al, 1999, Hu et al, 1999, Chevalier et al., 2002), the use of such VLPs as a vaccine has not been described. The same holds true for goose hemorrhagic polyoma virus (Zielonka et al., 2006) and Newcastle disease virus (McGinnes et al, 2010). Prel et al. (2007) described the co-expression of both AIV glycoproteins HA (H5), NA (N3) along with the matrix protein M1 in a baculovirus system. Although no formation of VLPs was observed the vaccination of Muscovy ducks induced protection from viral challenge. Later, the formation of VLPs was confirmed by the same group, but no vaccination and challenge experiments were not described (Prel et al., 2008). Recently, it has been described for H9N2 viruses that VLPs

consisting of the HA and M1 protein induced protective immunity in specific pathogen free (SPF) chickens and provided the possibility to use these vaccines for the DIVA (differentiating infected from vaccinated animals) approach due to the lack of presence of the influenza virus nucleoprotein (NP, Lee et al., 2011).

The aim of this study was to investigate if an influenza virus based VLP can be used for immunization of SPF chickens using the aerosolization of virus antigen. So far, no mass application method is available for vaccination of chickens against HPAI. The combination of aerosolization with the non-infectious influenza virus based VLP would allow for the DIVA approach and mass vaccination.

Material and Methods

Cells and Virus

Madin-Darby canine kidney (MDCK) *cells* (CRL-2285, ATCC, Manassas, VA, USA), were grown in Dulbecco's Modified Eagles's Medium with 4.5g/l glucose (DMEM-4.5, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Mediatech, Manassas, VA, USA). The insect cell lines of *Spodoptera frugiperda* cells (*Sf*9, Invitrogen) were cultivated in serum free SFX-Insect medium (Thermo Scientific, Waltham, MA, USA) at 28°C.

For the hemagglutination inhibition assay and also for the virus neutralization assay, a recombinant virus was used named H5N1-Garut which was provided by Dr David Suarez (USDA-ARS, Athens, GA). The virus was generated following the methods described before (Jadhao and Suarez, 2010). The cDNA of the HA segment was taken from the highly pathogenic avian influenza virus (HPAI) A/Chicken/Garut/BBVW-223/2007 (H5N1) while the cDNA for the NA was derived from the HPAI A/Chicken/Indo/07/03 (N5N1) virus isolate. The remaining

viral based cDNA segments were taken from the mouse adapted A/Puerto Rico/8/34(H1N1). The virus was propagated in the allantoic cavity of nine-day-old specific-pathogen free (SPF) embryonated chicken eggs (Sunrise Farms, Catskill, NY, USA) at 35°C for 4 days. The allantoic fluid was tested by hemagglutination (HA) assay (OIE, 2009) and those showing the highest HA titers were pooled, aliquoted and stored at -80°C until use.

Generation of an HA5-encoding recombinant baculovirus

The ORF of the H5 antigen encoding for the HA of A/Vietnam/1203/04 (H5N1) was codon optimized for human cells and synthesized (GenScript Inc., Piscataway, NJ). The nucleotide sequence encoding the polybasic HA cleavage site was not included to prevent cleavage during propagation by ubiquitous present cellular proteases and due to governmental regulations. To obtain a soluble protein that can be purified from infected Sf9 cells the C-terminal located transmembrane domain and cytoplasmic tail encoding sequence was truncated by PCR using two oligonucleotides (HA5-HisFP, cc**ACTAGT**ATGGAGAAAATAGTGCTTCTTCTTGC, HA5DTMD-RP, HA5-HisRP GG**AAGCTT**CAATGGTGATGGTGATGGTGTATTGATTCCAATTTTACTCCAC) and Deep Vent polymerase (New England Biolabs, Ipswich, MA, USA). The used restriction enzyme cleavage sites have been bold typed while the 6xHis coding sequence was underlined. The obtained PCR fragment was gel eluted using QIAquick gel Extraction kit (Qiagen, MD, USA). The eluted cDNA fragment was incubated with the restriction enzymes Spe I and Hind III, gel eluted again and ligated into the appropriately cleaved ***pFastBac***™ Dual baculovirus transfer vector (Invitrogen). Obtained recombinant plasmids were sequenced and one plasmid was selected (pFAST-H5Viet-His) for generation of a recombinant baculovirus using the Bac-to-Bac system (Invitrogen) following the instructions of the

manufacturer. The cell culture supernatant of transfected cells was harvested, passaged two times in Sf9 cells and the supernatant of the second passage was used for infection of Sf9 cells for subsequent protein purification.

Detection of serum antibodies by ELISA

Protein purification and detection of the purified protein was performed as described before (Dlugolenski et al, 2010). The purified recombinant H5His-Vietnam protein was used in an indirect ELISA. To this end, 96-well flat-bottom plates (Fisherbrand, Santa Clara, CA) were coated with H5His-Vietnam protein diluted 1:10 with coating buffer (KPL) which resulted in 250 ng/well. Plates were incubated at 4°C overnight, the supernatant removed and plates were washed three times with 100 µl of 1x wash solution (KPL). 100 µl of 1x blocking solution (KPL) was added, the plates were incubated for 1 hour at 37°C and after the removal of blocking solution the plates were washed three times. Serum samples were diluted 1:10 in sample dilution buffer (Synbiotics, Kansas City, MO), 50 µl of this diluted serum was added per well and the plates were incubated at 37°C for one hour. Now the serum dilution was removed, the plates washed again and 50 µl of goat anti-chicken IgG horseradish-peroxidae (HRP) conjugated antibodies (KPL) in a 1:500 dilution were added to each well. The plates were incubated at 37°C for one hour before the antibody conjugate was removed and plates were washed. Fifty microlitersof the HRP-substrate solution (KPL, Gaithersburg, MD) were added to each well and the plates were incubated at room temperature for 15 minutes. Finally, 50 µl of 1x stop solution (KPL) was added to each well and the OD value of each well was measured at 405 nm with an ELISA plate reader (ELx 808, BioTek, Winooski, VT).

Hemagglutination-Inhibition Assay

In order to determine hemagglutination inhibiting (HI) antibodies in chicken sera, the HI assay was performed. In general, the procedure followed the OIE recommendations (OIE, 2009). Briefly, SPF-eggs propagated LPAI H5N1-Garut virus was diluted in phosphate buffered saline (PBS) to 4 HA units. Twenty five microliters of PBS was pipetted into each well of a round-bottom, 96-well plate (Costar, Corning, NY) and 25 µl of a chicken serum sample was added to the first well and diluted two-fold down each row of the plate. After 25 µl of the diluted virus were added to each well, the plate was sealed with Linbro Plate sealer (MP Biochemicals, Solon, OH) and incubated at room temperature for 45 minutes. This was followed by the addition of 25 µl of 1% chicken red blood cells diluted in PBS and an incubation step of 40 min at room temperature. The highest serum dilution at which complete hemagglutination inhibition was observed was recorded as the HI titer.

Virus neutralization assay

The virus neutralization assay was performed in 12-well tissue culture plates. To this end, MDCK cells were seeded into the tissue culture plate's wells 24 hours prior to performing the assay in order to obtain a 100% confluence of the cells. The recombinant H5N1-Garut virus was diluted to a titer of 100 plaque forming units (PFU) per one hundred microliter in serum free DMEM-4.5 and kept on ice until use. In a 96-well tissue culture plate 50 µl of serum was added to 50 µl of DMEM-4.5 and diluted two-fold. Now to the 50 µl diluted serum the same volume of the diluted H5N1-Garut was added and the serum-virus mixture was incubated at 37°C for 1 h. A serum from an SPF chicken (Merial, Gainsville, GA) and a serum from a chicken which has been infected with a H5N1 wild bird isolate (Mundt et al, 2010) was used as negative and positive

control, respectively. Next, the medium was removed from the MDCK cells, the cells were rinsed once with serum free DMEM-4.5 and 500 µl of DMEM-4.5 was added into each well followed by the addition of the 100 µl serum-virus mixture. The plates were incubated 1 h at 37°C, the supernatant was removed and cells were rinsed with 1 ml of DMEM-4.5. Next, 1 ml of overlay medium [1.2% Avicel RC-581F (FMC BioPolymer, Philadelphia, PE), 1x MEM (Invitrogen), 40 mM HEPES, 2 mM L glutamine, 0.15 % of $NaHCO_3$, 100 IU penicillin/ml, 100 µg streptomycin/ml, 1 µg/ml TPCK-treated trypsin (Worthington Biochemical Corporation, Lakewood, NJ)] was added and the plates were incubated at 37°C. After 48 h of incubation the overlay was removed, cells were rinsed once with PBS and fixed with an ice-cold acetone/methanol mixture (60%/40%, v/v) for 10 min at room temperature. The cells were air-dried and 1 ml of Crystal-violet staining solution [0.065 % Crystal violet (w/v), 2.5 % methanol, 5.5 % formaldehyde, 0.5x PBS] was added and incubated for 10 min at room temperature. Finally the staining solution was removed, the cells were rinsed once with tap water and air-dried. The PFU were counted and a reduction of 80% of the PFU/well in comparison to the negative control serum was considered as positive virus neutralization. The test was only valid when the positive control serum showed a > 80% reduction of PFU.

Determination of respiratory parameters for chickens

For the application of virus-like particles via the aerosol route the respiratory parameters needed to be determined using plethysmography systems (EMKA Technologies, Falls Church, VA). These experiments focused on the determination of inhaled air volume and respiration frequency. To this end, one-day-old SPF chickens (Merial, Gainsville, GA) were placed into a respiratory chamber for rats (EMKA Technologies, Falls Church, VA) located in a dark room and the

respiratory parameters were measured. The lights in the room were dimmed to strongly reduce the excitation of the chickens and encourage normal breathing. The parameters were determined in two different sessions and for each session ten chickens were used. The recorded and subsequent calculated parameters were: inhaled air volume per breath, number of breaths per minute and elapsed time until 100 ml of air was inhaled.

Aerolization of virus like particles

The influenza A virus like particles (VLP) were provided by Dr Song (Emory University, Atlanta, GA) and were produced as described before (Song et al, 2010). Briefly, the VLP's contain the HA and M1 protein of A/Indonesia/05/2005 (H5N1) which were encoded by a recombinant baculovirus (rBV). The encoding sequence for the polybasic cleavage site of the HA protein was removed prior to generation of the rBV. Sf9 cells were infected and 3 days after infection the cell culture supernatants were clarified by centrifugation and then were concentrated by hollow fiber based filtration using Quixstand (GE Healthcare, Waukesha, WI). Sucrose gradient ultracentrifugation with layers of 20%, 30% and 60% (wt/vol) was performed for purification of VLPs at 28,000×g for 60 min. VLPs were mainly purified from a band between the 30% and 60% sucrose gradient. The presence of the H5 and M1 protein was confirmed by Western blot using an H5-specific Mab VN1203/02 (NR-2730; BEI Resources, Manassas, VA) and an influenza virus M1 specific Mab ab17265 (Abcam pic, Cambridge, UK), respectively. The VLPs were diluted in VLP dilution buffer (25 mM NaPi pH 7.2, 500 mM NaCl, 0.01% Tween 80) to a concentration which allowed the expected uptake of the correct amount of VLPs. The chickens were restrained in the aerosol chamber with airflow of 1 liter per minute. The conditions of the aerosolization were adjusted so that each chicken obtained the

same amounts of VLPs. After exposure, the chickens were removed from the chamber and held in Horsfall-Baur units at the Poultry Diagnostic Research Center (College Veterinary Medicine, University of Georgia, Athens, GA) with HEPA exhaust and supply air. Feed and water was supplied ad libitum. The chickens were bled via the brachial vein at different time points after vaccination in dependence of the experiment (see result section). The clotted blood was incubated over night at 4°C, centrifuged for 5 min at 2000x g, and the obtained serum sample was incubated at 56°C for 30 min prior to use in the appropriate assays. After the last bleed, the chickens were euthanized following the protocol (A2009 10-099-Y3-A0) approved by the University of Georgia Institutional Animal Care and Use Committee.

Results

Determination of the respiratory parameters of chickens

The determination of the respiratory parameters was a prerequisite for all subsequent experiments. The experiments were performed on one-day-old SPF chickens. Each experiment was performed on 10 chickens and three independent experiments were performed. Since such data were not available, the experimental set up needed to be optimized for chickens. To ensure sedation of the chickens, the experiments were performed in a room where the light was dimmed. The chosen set up led to the absence of any excitations of the chickens. Several chambers were tested (guinea pig, rat, ferret) and the rat chamber was chosen due to its size and applicability for use with 14-day-old SPF chickens. The obtained data are shown in table 1. One-day-old chickens breathed, on average, 72.8 times per minute. The inhaled air volume per breath was 0.76 ml. In addition, it was determined using software provided by EMKA technology how long it took for 100 ml of air to be inhaled. This value was determined to be 90 s per chicken.

Also, the value of for the inhaled air per minute (56.4 ml) was determined. Because the chickens exhibited relatively high variabilty for all four determined parameters, as expressed by the standard deviation (see table 1), a specific experimental set up was chosen and used on each bird. The membrane which facilitates the aerolization of the antigen was able to aerolize 2 ml of a watery solution into an aerosol with an average diameter of 2 μm per droplet in 90 s. Thus the concentration of the antigen was adjusted to a concentration that the chicken was able to inhale targeted amount of antigen while inhaling 100 ml of aerosol.

Establishment of an indirect H5-specific ELISA

Traditionally, hemagglutination inhibition (HI) assays as well as virus neutralization assays are used for the detection of serum antibodies specific for the influenza virus HA protein. Following the OIE recommendation for the detection of influenza virus subtype specific antibodies, the HI assay is the method of choice (OIE, 2009). Due to the novelty of the experiments, a third assay was employed which might detect a broader spectrum of antibodies besides neutralizing antibodies and HI antibodies. To this end, the H5-His protein of A/Vietnam/1203/04 (H5N1) was expressed in a baculovirus-based expression system and purified using Cobalt-ion based affinity chromatography (Fig 1). The protein was purified following the protocol as described before (Dlugolenski et al, 2010). The protein stained gel clearly showed a band with the expected size of 68 kD (Fig 1, left panel). To verify the identity of the protein a Western blot was performed using the H5-specific MAb VN1203/02 (NR-2730; BEI Resources, Manassas, VA). The result unequivocally showed that the obtained protein was H5-specific and only present in H5-baculovirus infected Sf9 cells (Fig 1, right panel). Purified H5-protein was pooled and glycerol was added up to one-fifth of the final volume. The protein solution was aliquoted at -20°C until

use. Since the indirect ELISA was intended to be used with SPF chicken sera, antigen dilutions were performed so that a serum dilution 1:10 of serum samples of naïve SPF chickens did not exceed an average OD value of 0.2. Using this approach, an antigen amount of 250 ng per well was used. Using this amount of recombinant protein, H5-specific antibodies were detected in serum samples from chickens which had been infected with LPAI from the North American lineage (H5N1, H5N2, H5N3) generated during infection experiments as described before (Mundt et al, 2009). Serum samples of infected SPF chickens which showed titers of one or two in the homologous HI test (H5N1 antigen versus serum samples from H5N1 infected chickens) tested positive in the indirect ELISA, although they would be considered as negative (OIE, 2009). This indicated that the indirect ELISA was sufficiently sensitive for the intended experimental approach using SPF chickens for the vaccination studies. Next the cut off value for the indirect ELISA was determined using 80 serum samples from SPF chickens at a serum dilution of 1:10. The average OD value was 0.224 with a standard deviation of 0.109. The cut off value was determined with 0.44 which resembles to the average plus two times the standard deviation.

Immunization of chickens with 100 µg of virus like particles induced seroconversion in all three assays

The VLPs were produced and purified as described in the material and methods section. The VLP consisted of the HA and M1 protein naturally encoded by A/Indonesia/05/2005 (H5N1) save for the nucleotide sequence encoding for the polybasic cleavage site was removed. The presence of the hemagglutinin H5 and M1 protein in the purified VLP preparation was tested by Western blot analysis (Fig 2) using the H5-specific Mab VN1203/02 and an influenza virus M1

specific Mab (Abcam pic, Cambridge, UK). The results of the Western blot proved the presence of the target proteins in the VLP preparation. In the first experiments, one-day-old SPF chickens were vaccinated with 100 μg of VLP per chickens in three routes: intramuscular, intranasal, and aerosol. For each vaccination no adjuvants were added to investigate the ability of the VLP to induce H5-specific antibodies. Prior to dilution, after dilution, and after aerolization the VLP preparations were tested for their ability to hemagglutinate chicken erythrocytes employing the HA test as described (OIE, 2009). In addition it was tested if during aerolization the HA activity of VLP changed. The undiluted VLP preparation containing 2 mg protein per milliliter showed an HA titer of 2^{17}. The VLP preparation was diluted 1:4 in VLP buffer (500 μg/ml) and showed a HA titer of 2^{11} before and 2^{9} HA units after aerolization. The VLP suspension was obtained after aerolization from the chamber since some of the VLPs containing aerosol precipitated in the chamber. In this experiment, conditions were chosen so that each chicken should inhale 100 μg of VLP (AE group). The same amount of VLP was given intranasally (IN group) and intramusculary (IM group) to one-day old SPF chickens. One group of chickens served as negative control group (Con) and received no VLPs. The serum samples obtained 14 days after the first vaccination were tested by three independent assays (VN test, HI test, indirect ELISA). Interestingly the HI test as well as the VN test showed a positive reactivity in the IM group (Fig 3). The remaining three groups (IN, AE, Con) showed no positive reaction. The results of the indirect ELISA supported the findings. Only the IM group showed a significant difference from the cut off (OD>0.45). Fourteen days after the first vaccination, chickens obtained a booster vaccination using the same route. The HA titers of the diluted VLP before and after aerolization were 2^{12} and 2^{10} HA units, respectively. The chickens were bled 14 d and 21 d after the second vaccination. The analysis of the serum samples from day fourteen after booster vaccination

showed an increase in HA titer and VN titer in the IM group (Fig 3, middle panel). Interestingly, the one chicken in the AE group showed an HI titer of 2^1, which is not significant in accordance to the OIE recommendations. Also, this serum sample and a second serum sample were positive in the VN test with a VN titer of 2^3 and 2^4, respectively. The results of the indirect ELISA showed the same trend. The IM and AE group showed significant positive OD values (>0.45) while the IN group showed again no significant positive results in either test. It needs to be mentioned that only one chicken in the IN groups (OD value of 0.55) was significant positive in the indirect ELISA. Three weeks after booster vaccination very similar results were observed (Fig 3, lower panel). The IM group showed very similar results compared to 14 d after booster vaccination, with the titers in all three tests showing no significant increase. Interestingly, the VN and HI titers in the AE group increased. Three out of five of the sera were positive in the HI test and all five were positive in the VN test. But these titers were very low and can only be considered as a trend. Again only one chicken in the IN group showed an HI titer of 2^1, which can be considered as negative. The sera from the control chickens remained negative in all three tests.

Lower amounts of VLP antigen did induce seroconversion in absence of an adjuvant only after intramuscular application

The next experiments were performed to test if a lower amount of antigen would also be able to induce an immune response in SPF chickens. The dilution of the antigen in VLP dilution buffer was chosen so that each chicken would inhale either 5 μg or 20 μg of VLP antigen. The HA titer of the diluted antigen (20 μg) was 2^7 before and 2^3 after aerolization while the antigen dilution for the 5 μg exposure resulted in an HA titer of 2^6 before and 2^3 after aerolization. The SPF

chickens were, as before, vaccinated at one day of age via the three routes described above. The chickens were bled at 14 days after the first vaccination and tested again in all three tests (HI test, VN test, indirect ELISA). All chickens vaccinated via the AE and IN route and the control chickens did not show any evidence of seroconversion (Fig 4 , upper panel). Chickens vaccinated via the IM route showed high OD values in the indirect ELISA. The group of chickens vaccinated IM with 5 µg and 20 µg showed an average OD value of 1.13 and 1.41, respectively. The HI test resulted in a titer of 2^3 in the serum of one chicken of the 5 µg group and two chickens in the 20 µg group (both 2^2). The VN test showed only one chicken of the 5 µg group with a titer of 2^2. This chicken was also positive in the HI test. The chickens were vaccinated again 14 days after the first vaccination with the aim to boost the immune response induced by the first vaccination. Again, 14 d after the boost vaccination the chickens were bled and the serum samples were analyzed (Fig. 4 lower panel). The serum samples of the chickens vaccinated via the AE and IN route again showed no positive HI and VN titers. In addition, the OD values obtained in the indirect ELISA were not significantly different from the cut off value of 0.44, and were thus labeled negative. Only one chicken in the 20 µg AE group showed an OD value of 0.554. The serum samples obtained from chickens of the IM groups showed an increase in HI and VN titers indicating a booster effect of the second vaccination. The HI titers ranged from zero to 2^7 in the 5 µg group and from 2^1 to 2^5 in the 20 µg group. The VN titers showed a similar trend as indicated by an increase of the average values in both groups. The booster vaccination did not increase the OD values of the group vaccinated with 20 µg. In contrast, the booster vaccination of the 5 mg group resulted in an increase of the average OD value from 1.13 (1st vaccination) to 1.65 (2nd vaccination).

Discussion

The ideal solution for vaccinating chicken flocks would be the use of attenuated, live AI vaccines of the appropriate subtype. However, the danger of mutations in the vaccine virus which might lead to higher pathogenicity and the possibility of reassortants which might endow these viruses with unpredictable properties prohibit this approach. Live vaccines are attractive because they would allow for mass vaccination as is performed with other poultry viruses. Vaccination of poultry is always the vaccination of extremely large amounts of animals due to the nature of industrial production methods. When the vaccines must be given either intramuscularly or subcutaneously to induce an efficient protection from virus challenge in the field or experimental settings, there is always the problem of a large amount of labor and time associated with these methods. Ideally, the vaccine provides a level of protection where neither clinical signs nor virus shedding can be observed. Inactivated vaccines using LPAI viruses that are antigenically similar to the HPAI they seek to protect against have been used for over 30 years (Swayne, 2009). The viruses for these vaccines are produced in embryonated chicken eggs, the infectivity of the virus is inactivated by chemical compounds and an emulsion vaccine is produced. Such vaccines induce very high antibody titers in poultry and the protection from homologous HA field strains is good over extended periods of time (Swayne et al, 1999). However, the necessary parenteral vaccine application induces a high cost of labor Moreover, it is almost impossible to detect infected birds by routine serological tests in the vaccinated population. The use of recombinant vaccines might be a viable approach, but currently registered vaccines based on recombinant fowlpox virus must also be given parenteral. Also, existing immunity to the vector virus diminishes the efficient replication of the vaccine virus and, thus, a sufficient protective immune response will not be developed by the vaccinated bird (Swayne, 2009). The advantage of a vector

vaccine is that the DIVA approach is a viable method since the highly immunogenic viral nucleoprotein can be used as target antigen for serological screening.

The use of recombinant proteins expressed in heterologous systems is on the rise with the availability of recombinant DNA technology,. Recombinant vaccines are on the market for Hepatitis B and papilloma virus infection in humans (Zuckermann, 1987; Harvengt, 1988). The latter was the first vaccine to prevent cervical cancer in humans (Speck and Trying, 2006, Bryan, 2007). The use of influenza viruses vaccine candidates based on VLP in poultry was poorly investigated (Prel et al.2007, Prel et al, 2008). Recently it was described that a single does of parenteral given H9N2 based VLP induced partial protection when administered alone and in conjuction with an adjuvant (Lee et al., 2011). A very important advantage of vaccines based on these new technologies is that they can be engineered to exclude the influenza A virus NP. Therefore, these vaccines can be used in the DIVA approach where vaccinated animals can be differentiated from infected due to the presence of NP antibodies (Swayne, 2009).

Aerosol vaccination in poultry has been tested for several viruses: Newcastle disease virus (Gough and Allan, 1974, Kleven et al, 1976), infectious bronchitis virus Winterfield et al., 1976), herpesvirus of turkey vaccine to prevent Marek's disease (Eidson and Kleven (1976), avian reovirus vaccine (Giambrone and Hathcock (1991), and infectious bursal disease virus (Banda et al, 2008). Some of them are currently used in the poultry industry (Newcastle disease virus, infectious bronchitis virus). The mass vaccination of poultry by aerosol in combination with new vaccine technologies has been tested in the described experiments. It has been shown for the first time that aerosol vaccination of chickens with VLP resulted in a measurable seroconversion in the birds. The one-day-old chickens were chosen since the experiments were aimed to investigate whether an aerosol vaccination could be performed in hatcheries under higher hygienic

conditions. The results show that the use of influenza A virus VLPs without any adjuvant induced an immediate immune response after a single intramuscular vaccination. Similar results have been reported in a duck study where crude cellular lysates of insect cells containing baculovirus-expressed avian influenza H5:N3:M1 were inoculated using the same route (Prel et al., 2007). Interestingly, during our experiments boost vaccination by the aerosol route with 100 μg of VLP antigen resulted in a measurable titer in all three tests. That has not been described before for poultry. Recently, de Geus et al. (2011) described that aerosol vaccination of poultry with an inactivated influenza A virus of the subtype H9N2 did not induce influenza virus specific antibodies in vaccinated chickens after a single round of vaccination. This is in agreement with our finding that a boost vaccination was required to observe measurable amounts of HA-specific antibodies. Interestingly, Toro et al (2010) showed that a single spray vaccination of one-day-old SPF chickens with replication incompetent H7-encoding human adenoviruses did not induce measurable H7-specific serum antibodies although *in ovo* vaccination with the same viruses did induce a robust antibody response in chickens. That means that an aerosol vaccination of one-day-old SPF chickens with VLP is possible, but a booster vaccination seems to be necessary regardless of which expression system has been used.

Surprisingly, no significant seroconversion was observed in chickens which have been vaccinated by the nasal route. Initially, that was supposed to be used as a positive control since experiments in mice where the same composition of VLP (HA and M protein) was administered intranasally (Kang et al, 2009) induced an immune response. Interestingly, intranasal installation of only 100 ng or 300 ng of HA equivalent induced a long lasting immunity in mice which protected them up to 30 weeks after the last vaccination. Even the prime-boost regime during our experiments did not result in any significant anti-HA directed antibody titers. The reason for this

different phenotype can only be explained by differences in effector cells which are responsible for the induction of an appropriate immune response in the upper respiratory tract of mice which likely are not present in chicken.

Aerosol vaccination of chickens with VLPs clearly seems to be dose dependent since neither 5 μg nor 20 μg per chicken was able to induce a measurable HA-specific antibody response in chickens. The dose dependence was also observed in the chickens which have been vaccinated via the intramuscular route since the HI titers after a single vaccination with the lower dosage was almost not detectable. The same was not observed in the indirect ELISA since independent from the dosage the ELISA OD values were of comparable readings even when the sera were analyzed in parallel on the same plate. This might be explained by the fact that the indirect ELISA detects a broad variety of antibodies to the HA, while the VN as well as the HI test detects a specific subset of antibodies in the sera of chickens.

In summary, the performed experiments show clearly, for the first time, that aerosol vaccination of chickens with influenza A virus based VLPs is possible when a boost vaccination is performed. This effect was clearly dependent on the amount of VLP.

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Table 1: Measured respiratory data for 1-day-old SPF chickens

Measured parameter	Average	Standard deviation
Breaths per minute	72.8	9.3
Inhaled air (ml)/breath	0.76	0.48
Inhaled air (ml)/min	56.4	14.7
Time (min) until 100 ml air were inhaled	1.5	0.27

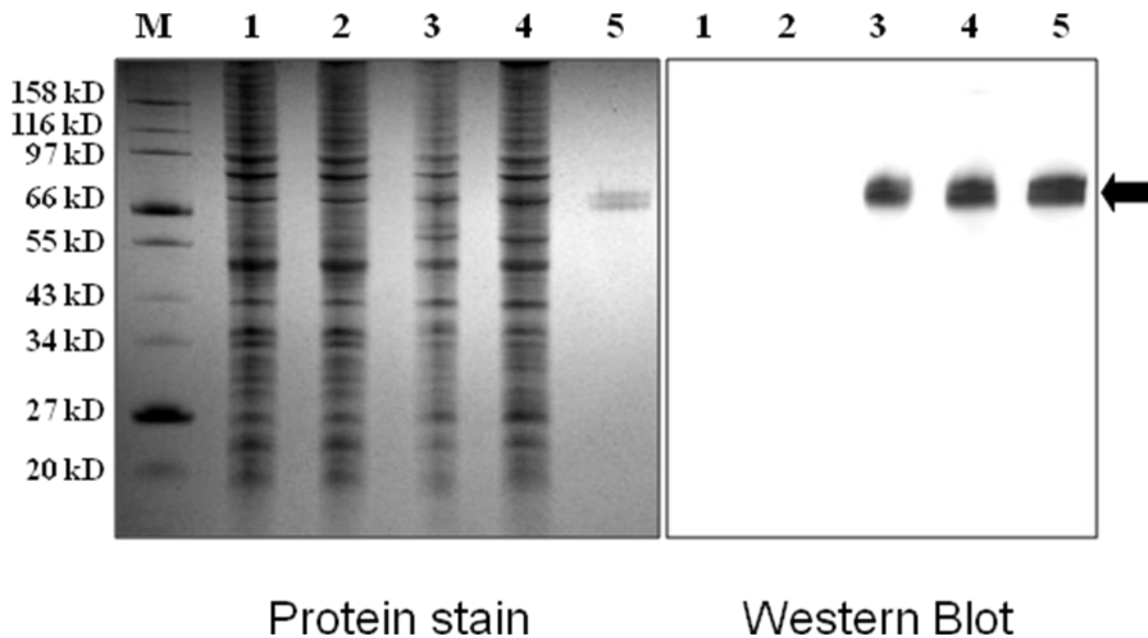

Figure 1. Purification of baculovirus-expressed H5His-Vietnam. Lysates of noninfected (lanes 1 and 2) and infected (lanes 3 and 4) were analyzed before (lanes 1 and 3) and after (Lanes 2 and 4) centrifugation. The purified protein was shown in lane 5 and was marked by an arrow. The protein samples were analyzed either by protein stain or by Western blot using the H5-specific monoclonal antibody (MAb) VN1203/02. The binding of the MAb was visualized by an anti-mouse HRP-conjugated goat antibodies followed by enhanced chemoluminiscence. A protein marker has been shown at the left side of the gel.

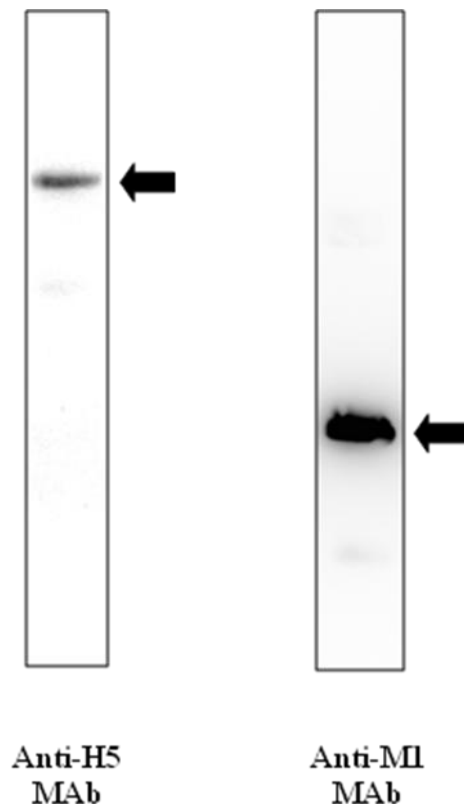

Figure 2: Virus like particles contain the hemagglutinin and M1 protein. A sample of virus like particles were separated by sodium dodecylsulphate (SDS)- polyacrylamide gelelectrophoresis (SDS-PAGE) on a 12.5% gel. The separated proteins were blotted on a nylon membrane and two lanes of the blotted membrane were exposed either to an H5-specific monoclonal antibody (MAb VN1203/02) or the M1 specific MAb ab17265. The binding of the MAb was visualized by anti-mouse HRP-conjugated goat antibodies followed by enhanced chemoluminiscence. The detected proteins were highlighted by an arrow.

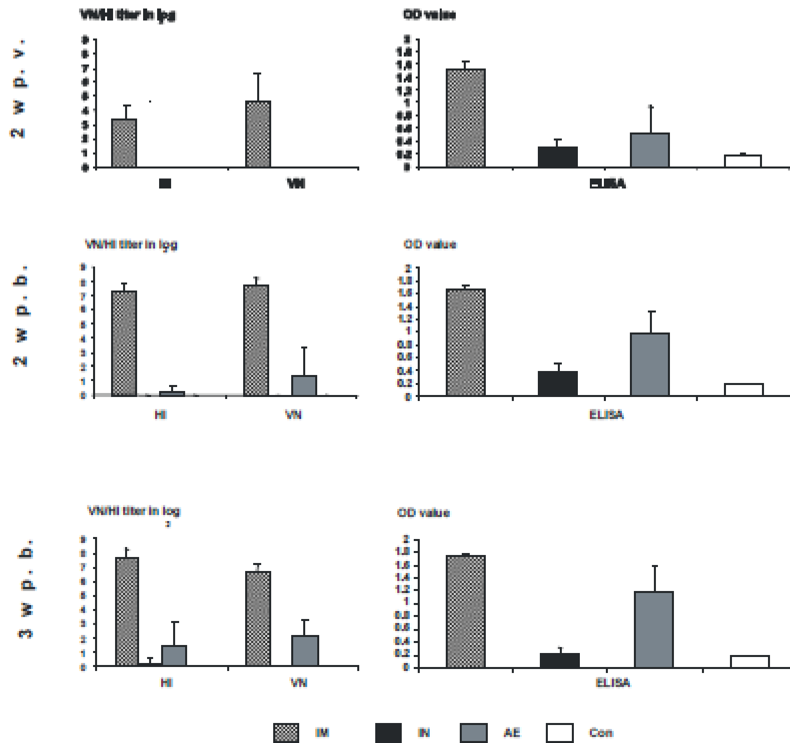

Figure 3: Analysis of the antibody response after vaccination with 100 µg virus like particles. One-day old SPF chickens were vaccinated via the intramuscular (IM), intranasal (IN), and aerosol route (AE). A group of chickens were not vaccinated and served as control (Con). Chickens were bled either at 14 d after vaccination (2 w p. v.) or 14 d and 21 d after the boost vaccination (2 w p. b.; 3 w p. b.). The serum samples were analyzed by hemagglutination inhibition (HI) assay using chicken red blood cells or virus neutralization (VN) test. Furthermore an indirect ELISA (ELISA) was performed using the purified H5His-Vietnam protein. The measures values are plotted on the Y-axis as the reciprocal value of the log 2 of the dilution where either no cythopathic effect (VN) or agglutination (HI) was observed. For the ELISA the OD value at 405 nm has been shown.

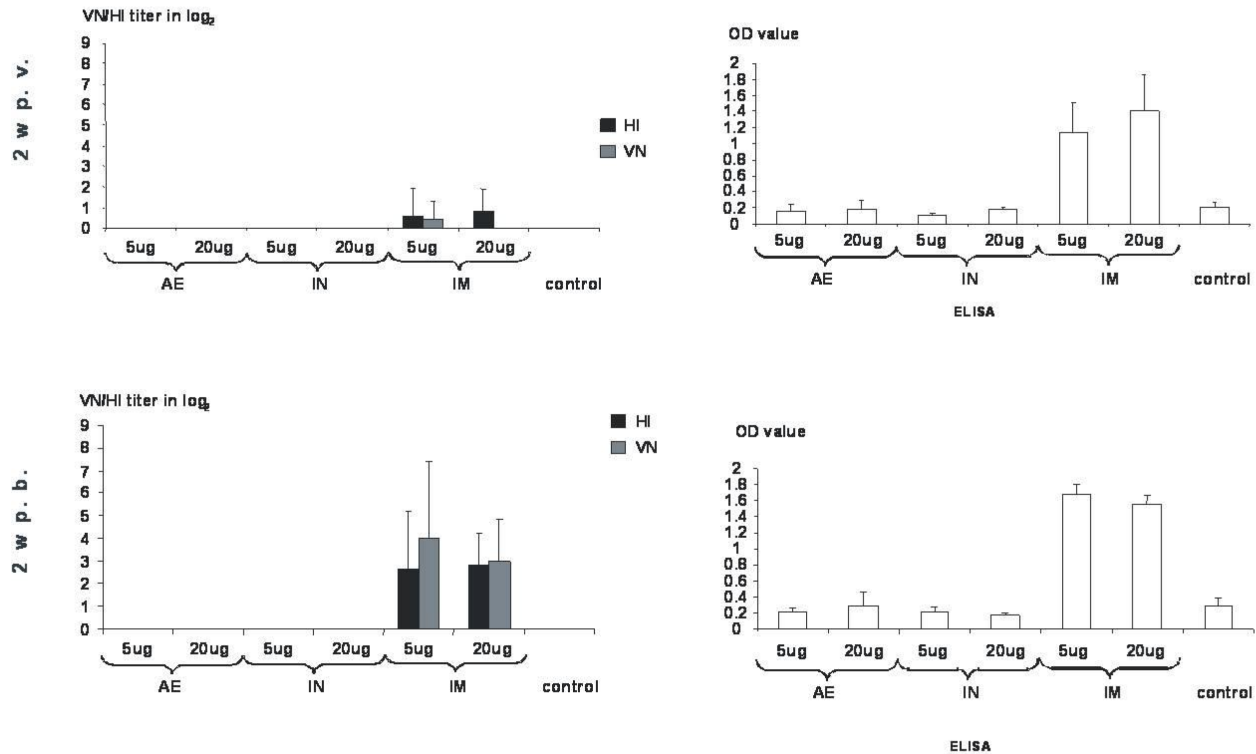

Figure 4: Analysis of the antibody response after vaccination with 5 μg and 20 μg virus like particles. One-day old SPF chickens were vaccinated via the intramuscular (IM), intranasal (IN), and aerosol route (AE). A group of chickens were not vaccinated and served as control (control). Chickens were bled either at 14 d after vaccination (2 w p. v.) or 14 d after the boost vaccination (2 w p. b.). The serum samples were analyzed by hemagglutination inhibition (HI) assay using chicken red blood cells or virus neutralization (VN) test. Furthermore an indirect ELISA (ELISA) was performed using the purified H5His-Vietnam protein. The measures values are plotted on the Y-axis as the reciprocal value of the log 2 of the dilution were either no cythopathic effect (VN) or agglutination (HI) was observed. For the ELISA the OD value at 405 nm has been shown.

CHAPTER 4

DISCUSSION

The ideal solution for vaccinating chicken flocks would be the use of attenuated, live AI vaccines of the appropriate subtype. However, the danger of mutations in the vaccine virus which might lead to higher pathogenicity and the possibility of reassortants which might endow these viruses with unpredictable properties prohibit this approach. Live vaccines are attractive because they would allow for mass vaccination as is performed with other poultry viruses. Vaccination of poultry is always the vaccination of extremely large amounts of animals due to the nature of industrial production methods. When the vaccines must be given either intramuscularly or subcutaneously to induce an efficient protection from virus challenge in the field or experimental settings, there is always the problem of a large amount of labor and time associated with these methods. Ideally, the vaccine provides a level of protection where neither clinical signs nor virus shedding can be observed. Inactivated vaccines using LPAI viruses that are antigenically similar to the HPAI they seek to protect against have been used for over 30 years (Swayne, 2009). The viruses for these vaccines are produced in embryonated chicken eggs, the infectivity of the virus is inactivated by chemical compounds and an emulsion vaccine is produced. Such vaccines induce very high antibody titers in poultry and the protection from homologous HA field strains is good over extended periods of time (Swayne et al, 1999). However, the necessary parenteral vaccine application induces a high cost of labor Moreover, it is almost impossible to detect infected birds by routine serological tests in the vaccinated population. The use of recombinant vaccines might be a viable approach, but currently registered

vaccines based on recombinant fowlpox virus must also be given parenteral. Also, existing immunity to the vector virus diminishes the efficient replication of the vaccine virus and, thus, a sufficient protective immune response will not be developed by the vaccinated bird (Swayne, 2009). The advantage of a vector vaccine is that the DIVA approach is a viable method since the highly immunogenic viral nucleoprotein can be used as target antigen for serological screening.

The use of recombinant proteins expressed in heterologous systems is on the rise with the availability of recombinant DNA technology,. Recombinant vaccines are on the market for Hepatitis B and papilloma virus infection in humans (Zuckermann, 1987; Harvengt, 1988). The latter was the first vaccine to prevent cervical cancer in humans (Speck and Trying, 2006, Bryan, 2007). The use of influenza viruses vaccine candidates based on VLP in poultry was poorly investigated (Prel et al.2007, Prel et al, 2008). Recently it was described that a single does of parenteral given H9N2 based VLP induced partial protection when administered alone and in conjuction with an adjuvant (Lee et al., 2011). A very important advantage of vaccines based on these new technologies is that they can be engineered to exclude the influenza A virus NP. Therefore, these vaccines can be used in the DIVA approach where vaccinated animals can be differentiated from infected due to the presence of NP antibodies (Swayne, 2009).

Aerosol vaccination in poultry has been tested for several viruses: Newcastle disease virus (Gough and Allan, 1974, Kleven et al, 1976), infectious bronchitis virus Winterfield et al., 1976), herpesvirus of turkey vaccine to prevent Marek's disease (Eidson and Kleven (1976), avian reovirus vaccine (Giambrone and Hathcock (1991), and infectious bursal disease virus (Banda et al, 2008). Some of them are currently used in the poultry industry (Newcastle disease virus, infectious bronchitis virus). The mass vaccination of poultry by aerosol in combination with new vaccine technologies has been tested in the described experiments. It has been shown for the first

time that aerosol vaccination of chickens with VLP resulted in a measurable seroconversion in the birds. The one-day-old chickens were chosen since the experiments were aimed to investigate whether an aerosol vaccination could be performed in hatcheries under higher hygienic conditions. The results show that the use of influenza A virus VLPs without any adjuvant induced an immediate immune response after a single intramuscular vaccination. Similar results have been reported in a duck study where crude cellular lysates of insect cells containing baculovirus-expressed avian influenza H5:N3:M1 were inoculated using the same route (Prel et al., 2007). Interestingly, during our experiments boost vaccination by the aerosol route with 100 µg of VLP antigen resulted in a measurable titer in all three tests. That has not been described before for poultry. Recently, de Geus et al. (2011) described that aerosol vaccination of poultry with an inactivated influenza A virus of the subtype H9N2 did not induce influenza virus specific antibodies in vaccinated chickens after a single round of vaccination. This is in agreement with our finding that a boost vaccination was required to observe measurable amounts of HA-specific antibodies. Interestingly, Toro et al (2010) showed that a single spray vaccination of one-day-old SPF chickens with replication incompetent H7-encoding human adenoviruses did not induce measurable H7-specific serum antibodies although *in ovo* vaccination with the same viruses did induce a robust antibody response in chickens. That means that an aerosol vaccination of one-day-old SPF chickens with VLP is possible, but a booster vaccination seems to be necessary regardless of which expression system has been used.

Surprisingly, no significant seroconversion was observed in chickens which have been vaccinated by the nasal route. Initially, that was supposed to be used as a positive control since experiments in mice where the same composition of VLP (HA and M protein) was administered intranasally (Kang et al, 2009) induced an immune response. Interestingly, intranasal installation

of only 100 ng or 300 ng of HA equivalent induced a long lasting immunity in mice which protected them up to 30 weeks after the last vaccination. Even the prime-boost regime during our experiments did not result in any significant anti-HA directed antibody titers. The reason for this different phenotype can only be explained by differences in effector cells which are responsible for the induction of an appropriate immune response in the upper respiratory tract of mice which likely are not present in chicken.

Aerosol vaccination of chickens with VLPs clearly seems to be dose dependent since neither 5 µg nor 20 µg per chicken was able to induce a measurable HA-specific antibody response in chickens. The dose dependence was also observed in the chickens which have been vaccinated via the intramuscular route since the HI titers after a single vaccination with the lower dosage was almost not detectable. The same was not observed in the indirect ELISA since independent from the dosage the ELISA OD values were of comparable readings even when the sera were analyzed in parallel on the same plate. This might be explained by the fact that the indirect ELISA detects a broad variety of antibodies to the HA, while the VN as well as the HI test detects a specific subset of antibodies in the sera of chickens.

In summary, the performed experiments show clearly, for the first time, that aerosol vaccination of chickens with influenza A virus based VLPs is possible when a boost vaccination is performed. This effect was clearly dependent on the amount of VLP.