Development of universal Influenza vaccine in chicken with insights on the extracellular domain of Matrix protein 2

DISSERTATION

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Abstract

Avian influenza (AI) is an infectious disease of avian species caused by type A influenza viruses with a significant economic impact on the poultry industry. In addition to affecting poultry, different subtypes of AI viruses can infect other species, thus complicating prevention and control. Vaccination is one of the main disease prevention strategies in many countries worldwide. Current influenza vaccines based on the highly variable hemagglutinin (HA) protein can provide effective protection against specific, antigenically matching virus, but little protection against more distant strains, even those belonging to the same subtype, and they do not provide heterosubtypic immunity. Therefore, to protect chickens against new strain of AI virus, as well as control and prevent virus spread among farms, new vaccines needed to be designed to overcome the limitations of conventional vaccines. One of the approaches for new vaccine design is targeting conserved regions of the influenza genome as possible universal vaccines to induce cross protective immunity against different strains and to eliminate constant vaccine updates based on circulating virus. The extracellular domain of ion channel M2 protein (M2e) is highly conserved among different AI strains suggesting that it would be a promising candidate for developing a universal influenza vaccine. Due to poor immunogenicity of the small M2e, various M2e conjugating carriers and adjuvant formulations have been used to enhance its immunogenicity and protective efficacy. In
this study, our goal is to evaluate the potential use of M2e-based vaccines for the control of AI in chickens.

In the first part of the study, a recombinant avian M2e protein expressed on the surface of the norovirus P particle (M2e-PP) was tested for its immunogenicity and protective efficacy against challenges with 3 different AI viruses in chickens. Two-week-old specific pathogen free chickens were vaccinated 3 times with M2e-PP either subcutaneously (SQ) with oil adjuvant or transmucosally (intranasal, IN; eye drop, ED; microspray, MS) without adjuvant. M2e-PP vaccination via the SQ route induced significant IgG antibody responses which were increased by each booster vaccination. Neither IgG nor IgA responses were detected from sera nor nasal wash of transmucosally immunized birds. Upon intranasal challenge, M2e-PP vaccination via the SQ route significantly reduced virus shedding from both the trachea and the cloaca for all three challenge viruses. Despite the absence of detectable IgG and IgA responses in birds vaccinated with the M2e-PP via the IN route, a similar level of reduction in virus shedding was observed in the IN group compared to the SQ group. In conclusion, M2e-PP vaccination in chicken has demonstrated its high immunogenicity and its ability to protect chickens against challenge with 3 different AI virus subtypes.

A combination of M2e-PP recombinant protein with inactivated influenza vaccine (IIV) was tested in chickens, as an approach to overcome the limited strain specific protection of the IIV. Co-immunization of birds with both vaccines did not affect production of M2e specific IgG antibody compared to the M2e-PP alone vaccinated
group. However, the co-immunized birds showed significantly higher hemagglutination inhibition antibody titers against vaccine and challenge viruses as well as cross reactive antibody responses against the H5, H6, and H7 viruses compared to the IIV alone vaccinated group. Upon intranasal homologous and heterologous virus challenges, combined vaccine groups showed greater reduction in viral titers from tracheal swabs compared to those groups receiving IIV alone. Moreover, M2e-PP antisera from vaccinated birds were able to bind to their native M2e target expressed on whole virus and infected cells and to inhibit viral replication.

Overall, our results supports that the universal vaccine approach using an M2e based vaccine can provide cross-protection against challenge viruses among different HA subtypes. Additionally, supplementing IIV with M2e-PP can expand the vaccine protective efficacy. Further improvements of the vaccine immunogenicity and efficacy are required for this vaccine to be practical. A better understanding of the protective immune mechanism will also be critical for the optimization of an M2e-based vaccine in chickens.
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Dedication

To the memory of my mother, and to my father, my sisters, my wife, and my sons (Youssef and Yehia)
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Chapter 1: Literature Review

1.1. Introduction

Influenza is a highly contagious disease causing serious health and economic threats to both humans and animals worldwide. Influenza viruses are enveloped viruses belonging to the family Orthomyxoviridae that includes six different genera; Influenza A, Influenza B, Influenza C, Isavirus, Thogotovirus and Quranflivirus (Shaw and Palese, 2013). Recently, a novel influenza virus, tentatively classified as Influenza D virus, was identified in swine, cattle, sheep, and goats. Among these hosts, cattle may be the natural reservoir of virus (Ferguson et al., 2016).

Influenza A viruses (IAV) are able to infect different animal species, including pigs, horses, sea mammals and birds, in addition to humans (Alexander and Brown, 2000). IAV occasionally produce devastating pandemics which include; 1918 (Spanish flu, H1N1), 1957 (Asian flu, H2N2), 1968 (Hong Kong flu, H3N2), and 2009 pandemic H1N1 (Capua and Alexander, 2004; Cohen, 2009). The estimated economic impacts of seasonal human influenza are also vast. In the U.S. alone, the seasonal influenza resulted in 25.34 million human cases a year which resulted in an annual economic cost of approximately $29.12 billion from the loss of productivity due to work absenteeism and death or from indirect medical costs (Mao et al., 2012). The severity of influenza disease can vary widely and is determined by several factors including the virulence of
circulating viruses, transmissibility and the immune status of the affected population, the season, how well the vaccine is working to protect against illness, and how many people were vaccinated (Alexander and Brown, 2000).

In veterinary and agriculture sectors, serious economic consequences of avian influenza (AI) outbreaks have been experienced. For instance, outbreak of H5N2 highly pathogenic AI (HPAI) in Pennsylvania in the early 1980s resulted in more than 17 million culled birds, including chickens and turkeys, and a cost of more than $500 million to eradicate and control the outbreak. The HPAI H7N1 outbreak in the Italy (1999-2000) resulted in culling of 18 million birds with $100 million in compensation to affected farmers and $500 million in indirect losses. Another AI outbreak in Virginia in 2002 resulted in slaughter of 4.7 million birds and economically impacted the industry with approximately $130 million. The HPAI H7N3 outbreak in Mexico in 2012 affected a region where approximately 55% of the table eggs are produced and more than $22 million in losses were reported by the industry (U.S. Department of Agriculture, 2014). Recent HPAI outbreaks in the U.S. between December 2014 and June 2015 resulted in 223 cases in domestic poultry flocks in 15 states which became the most severe epizootic event in the history of the poultry industry. This incident affected almost 42.1 million table egg laying hens and pullets and 7.5 million turkeys. The cost of this HPAI outbreaks resulted in $3.3 billion in losses to the poultry industry which was the highest economic impact to date (Jennelle et al., 2016). Earlier this year, novel H7N8 HPAI
viruses were detected in commercial turkey flocks in Indiana (USDA, 2016). In addition to their impact on animal industries, the transmission of AI viruses to humans has demonstrated the significant zoonotic potential of the viruses (Melidou et al., 2009). Thus, controlling circulation of the AI viruses in poultry is essential for reducing the risk of human infection. Given the persistence of those viruses in some poultry populations, control will require long-term commitments from countries and strong coordination between animal and public health authorities.

Effective vaccination programs for influenza can lead to a robust immune response that can reduce clinical disease symptoms, reduce the amount of virus that is excreted from an infected host, and increase the resistance of the host for infections. All these factors can help break the transmission chain that can end an epidemic (Lee and Suarez, 2005). The rapid development of IAV vaccines and associated reference reagents is an important part of preparing for a disease outbreak caused by a new and emerging animal IAV with epizootic and/or pandemic potential. However, developing influenza vaccines for new and emerging strains presents significant challenges due to several factors related to virus itself (the genetic and antigenic diversity), host (preexisting immunity), co-infection with other pathogens, and process of vaccine manufacture (Subbarao and Joseph, 2007).
Herein, we will provide brief introduction about IAV (etiology, replication, and viral proteins) and different types of AI vaccines with focus on one of the universal vaccine targets, extracellular domain of Matrix protein 2 (M2).

1.2. Influenza A virus (IAV)

Etiology

IAV are classified into different subtypes based on the antigenicity and phylogenetic clustering of their hemagglutinin (HA) and neuraminidase (NA) proteins (Gioia et al., 2008). Till now, 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11) have been identified (Tong et al., 2013).

An infectious IAV particle consists of a spherical or pleomorphic lipid bilayer membrane, 80-120 nm in diameter, derived from the host cell during the budding step. On its surface, there are three membrane bound proteins including HA, NA, and Matrix protein 2 (M2). By proportion, 80% of the surface proteins are HA, around 17% are NA, and the remaining three percent is M2, with only 16-20 molecules per virion. Directly underneath the lipid bilayer is a scaffold of matrix 1 (M1) protein that connects the surface proteins and the viral ribonucleoprotein complexes (vRNPs), which provides the overall structure of the viral particle. Inside the virion, the eight vRNPs consist of negative sense RNA segments that are completely bound by the nucleoprotein (NP) with the tripartite viral RNA polymerase complex (polymerase acidic; PA, polymerase basic 1 ;PB1, and polymerase basic 2 ;PB2) on the 3’ terminus (Skehel and Wiley, 2000).
Viral proteins

IAV genome, ~ 13 kb in size, is made up of eight segments of negative sense single stranded RNA encoding for up to 17 proteins (Shi et al., 2014). Influenza proteins encoded by each segment are as follows:

1. 1st, 2nd, and 3rd segments encode PA, PB1, and PB2, respectively forming the polymerase complex. Segment 2 also encodes PB1-F2 and PB1-N40 by using alternative translation initiation sites (Chen et al., 2001; Wise et al., 2009). Moreover, segment 3 encodes PA-X by a ribosomal frame shift, as well as two additional N-terminally truncated forms (PA-N155 and PA-N182) by alternative translation initiation sites (Jagger et al., 2012; Muramoto et al., 2013).

2. HA transcript is on the 4th segment.

3. NP transcript is on the 5th segment.

4. NA transcript is on the 6th segment.

5. M1 transcript is on the 7th segment. It also encodes M2 and M42 by alternative splicing of M1 transcript.

6. Segment 8 encodes the nonstructural 1 (NS1) protein, nuclear export protein (NEP), and NS3 by alternative mRNA splicing.

Those viral proteins can be schematically fall in three classes: (i) structural proteins as HA, NA, M2, M1 and vRNPs; (ii) non-structural proteins as NS1 and NEP; (iii) nonessential or accessory proteins as PB1-F2, PB1-N40, PA-X, PA-N155, PA-N182.
(Dubois et al., 2014; Marc, 2014). Here we briefly describe each protein and its specific role.

**Structural viral proteins**

1. **HA: Receptor binding protein**

HA is a trimeric rod-shaped molecule with their carboxyl end inserted in the viral membrane and amino hydrophilic end spikes away from the viral surface (i.e., a type I integral membrane protein) (Shaw and Palese, 2013). As mentioned earlier, there are different HA subtypes which can be further divided into two antigenically-distinct groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15) according to their phylogenetic relationships based on the amino acid (aa) sequences (Laursen and Wilson, 2013).

The HA molecule exists as a homo-trimer in its native form, and each monomer consists of two polypeptides known as HA1 and HA2 (Shaw and Palese, 2013). The HA is first synthesized as a precursor polypeptide, HA0. Low pH environment makes its structure change, and thus HA protein is susceptible to host protease. The cleavage of HA into subunits HA1 and HA2 is an essential requirement for viral fusion and infectivity of the virus particle, and also plays an important role in the pathogenic properties of the viruses (Klenk et al., 1975). HA proteins of human influenza and low pathogenic AI (LPAI) viruses have a single arginine at the HA cleavage site, and can be cleaved by trypsin-like protease present in bird intestinal tract or human and bird respiratory tract
(Alexander, 2000; Horimoto et al., 1994). However, most HPAI viruses possesses multiple basic aa at the HA cleavage site, making it accessible to a wide variety of subtilisin-like intracellular proteases (Bosch et al., 1981; Webster et al., 2006a), while LPAI viruses are not susceptible to these proteases (Bosch et al., 1981). Once precursor HA0 is cleaved into HA1 and HA2, the newly exposed N-terminal of the HA2 peptide acts to fuse the viral envelope to the cellular membrane of the host cell. This acidic pH-mediated change makes the fusion peptide aligned antiparallel to the membrane anchor of the HA2, bringing the endosomal membrane into juxtaposition with the viral membrane, leading to fusion. The presence of more than one hemagglutinin then leads to the formation of a fusion pore through which the RNP can enter the cytoplasm and start viral replication (Shaw and Palese, 2013).

HA is responsible for binding IAV to the host cells as the receptor-binding site lies within the globular head of the molecule. It is well known that HA proteins of AI viruses preferentially bind to α2, 6-linked sialic acid (SA), whereas human and other mammalian viruses preferentially bind to α2, 3-linked SA (Connor et al., 1994; Rogers and Paulson, 1983). HA is also responsible for the determination of host range and tissue tropism. In humans, the α2,6-linked SA is dominant on epithelial cells in nasal mucosa, and it is believed that the low prevalence of α-2,3-linked SA molecules on the epithelial cells of the human upper respiratory tract decreases the susceptibility of humans to infection by AI virus (Shinya et al., 2006). In contrast, chicken and duck intestine express
the α2, 3-linked SA receptor type across the epithelial lining of villi (Ito and Kawaoka, 2000; Wan and Perez, 2006). In chicken tracheal epithelium, α2, 6-linked SA is the dominant receptor type whereas in ducks the α2, 3-linked SA receptor is more abundant in the ciliated cells of the tracheal epithelium and it was found that the ratio of α2,6-linked SA to α2,3-linked SA in chicken trachea was approximately 10:1 whereas in duck the ratio was 1:20 (Nicholls et al., 2007).

In addition to its role during viral replication, the HA is also the major determinant recognized by the adaptive immune system of the host. Following infection and replication, a vigorous immune response is triggered to the HA, the most abundant protein on the viral envelope, resulting in the formation of neutralizing antibodies (Shaw and Palese, 2013).

2. **NA: Viral sialidase glycoprotein**

The NA is the second most abundant glycoprotein expressed on the viral surface. It is a type II integral membrane protein with its N-terminus oriented toward the interior of the virus (Colman, 1994). The nine subtypes of NA are subdivided into two phylogenetic groups based on sequence comparison. The first group consists of N1, N4, N5 and N8 subtypes, and the second one consists of N2, N3, N6, N7 and N9 subtypes (Fouchier et al., 2005; Russell et al., 2006). Recently, novel NA subtypes were identified from bats (N10 and N11) which seem to be distantly related to existing subtypes and
display no apparent sialidase activity warranting the formation of a third NA group (Tong et al., 2013).

The NA is composed of four identical polypeptides arranged as homo-tetramer; each monomer is arranged into four domains; an N-terminal cytoplasmic domain, a hydrophobic transmembrane region, a thin hypervariable stalk, and a globular head domain that houses the enzymatic active site for the protein (Air, 2012). Furthermore, both enzymatic site and globular head are remarkably conserved across the majority of subtypes in contrast to the stalk region (Sylte and Suarez, 2009).

The NA is responsible for a plethora of functions during viral infection. It had a role early in infection facilitating entry of the virus (Matrosovich et al., 2004) and/or enhancing late endosome/lysosome trafficking (Suzuki et al., 2005). Another role is the cleavage of the α2-6 and α2-3 linked SA receptors from the surface of the cell and from the virus particles to release the virus from the infected cell and allow virus spread (Palese and Compans, 1976; Palese et al., 1974). In the absence of NA activity, IAV can infect and fully carry out one replication cycle, but progeny viruses remain aggregated on the host cell surface and thus fail to spread to uninfected cells (Marcelin et al., 2012).

Although the humoral response to HA is best characterized to protect against influenza infection (Webster et al., 1968), antibodies directed against the enzymatic site in the globular head region, as well as non-enzymatic globular head and stalk sites, show neuraminidase inhibitory activity and reduce disease mortality (McNulty et al., 1986;
Webster et al., 1988). While anti-NA antibodies are thought to be non-neutralizing, they may conceivably alter any of the other previously mentioned roles of NA in IAV infection. Anti-NA antibodies bound to the surface of infected cells may aid in viral clearance by immune effector cells via antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (Thomas et al., 2006). Thus, immunization with NA preparations has been proposed as an infection-permissive, disease-suppressive vaccine approach against influenza (Kilbourne et al., 2004).

3. Matrix proteins (M1 and M2)

M1: Scaffolding protein

M1 is the most abundant protein localized within the virion which lies just beneath the lipid envelope. The M1 protein is a collinear transcription product of Matrix gene which is different from the mechanism encoding the M2 protein. M1 is believed to make contact with the surface glycoproteins and with the vRNPs complexes, forming a bridge between the inner core components and the envelope proteins (Nayak et al., 2004).

The M1 protein has significant roles in establishing the virion assembly sites, nuclear export of vRNPs complexes, and viral budding (Barman et al., 2001; Noton et al., 2007; Rossman et al., 2010; Schmitt and Lamb, 2005). After translation in the cytoplasm, M1 proteins localize at either the apical plasma membrane or the nucleus (Barman et al., 2001). Localization of M1 at the apical plasma membrane is occur through interactions of M1 with lipids and the cytoplasmic exposed portions of HA, NA, and M2; thus allowing
for M1 to travel along with the exocytic membrane vesicles (Nayak et al., 2004). Once at the apical plasma membrane, M1 protein subunits will begin to interact with each other to form a scaffolding matrix for assembly of new viral particles (Chen et al., 2007). The interactions with both the surface proteins and other M1 subunits result in bringing the three surface proteins of the influenza viral particles closer together and in defining areas where the influenza particle will assemble and bud from the cell. If the M1 monomers do not interact with the viral surface glycoproteins, the M1 protein will localize at the nucleus through its nuclear localization signaling (NLS) sequence. Thus, M1 will interact with the vRNPs and NEP, which will allow for export of vRNP-M1-NEP complexes to the cytoplasm (Akarsu et al., 2003). M1 has also been shown to be necessary and sufficient for the formation of virus like particles, providing evidence for its essential role in the budding process (Gómez-Puertas et al., 2000; Latham and Galarza, 2001).

**M2: Proton ion channel**

The M2 protein of IAV is a tetrameric type III integral membrane protein (lacking a signal peptide sequence). M2 is encoded by a spliced mRNA of Matrix gene (Lamb et al., 1985; Martin and Helenius, 1991). It is composed of 3 domains: ectodomain, transmembrane, and endodomain. M2 has ion channel activity through its transmembrane domain, and is also involved in viral assembly and morphogenesis through its cytoplasmic domain (Iwatsuki-Horimoto et al., 2006; Lamb et al., 1985; Pinto et al., 1992; Wang et al., 1994). The protein forms a proton-selective ion channel, which is activated
at acidic pH (Pinto et al., 1992). During virus entry, M2 transports protons across the viral membrane, reducing the pH of the virion interior (Betakova, 2007; Bui et al., 1996), the acidic pH induces a dissociation of the M1 protein from the vRNPs, which is essential for entry of vRNPs in the nucleus of the infected cell (Betakova, 2007; Bui et al., 1996; Whittaker et al., 1996). M2 has also been implicated in stabilizing HA in the trans-Golgi network, the ion channel activity of M2 modify the acidic environment of the trans-golgi, preventing premature acid activation of newly produced HA (Betakova, 2007; Ciampor et al., 1992b). M2 is also involved in assembly and budding (Schroeder et al., 2005).

M2 is a specific target of the anti-influenza drugs amantadine and rimantadine which affect two steps of virus replication: virus uncoating and virus maturation (Betakova, 2007). Recently, the external portion of M2 has been considered the basis of a universal influenza vaccine approach as it maintains a highly conserved sequence over long periods of time which will be discussed further in later section.

4. **Viral ribonucleoprotein complex associated proteins**

Viral ribonucleoprotein complexes (vRNPs) refer to the replicative units that are comprised of a single segment of RNA and the bound proteins: including NP and polymerase complex (PB1, PB2 and PA) (Klumpp et al., 1997).

**Nucleoprotein (NP)**

NP is one of major protein components of vRNPs as it associates with viral RNA (vRNA) and the polymerase complex to form the ribonucleoprotein complex (Pons et al.,
1969). It plays an essential structural role in encapsidating the virus genomes into vRNPs. NP protein also directs nuclear import of the genome and is essential for vRNA synthesis in the nucleus (O’Neill et al., 1995). NP encapsulates the genome in a sequence-independent manner and interactions with the viral polymerase for viral RNA synthesis (Albo et al., 1995; Beaton and Krug, 1986). Subunits of NP interact with each other and with RNA to form a helical structure (Lamb et al., 1981; Shi et al., 1996). NP also interacts with Matrix 1 protein, PB1 and PB2 (Biswas et al., 1998; Ye et al., 1999). In addition to its function in RNA synthesis, a temperature-sensitive mutant of NP has been recently used to identify a late role for NP in virion assembly. Progeny viruses of NP mutant exhibited an abnormal morphology but virus genome transcription, replication, and protein synthesis were not affected (Noton et al., 2009).

**Polymerase basic 2 (PB2)**

PB2 is a component of the RNA dependent RNA polymerase complex (Toyoda, 1997). PB2 is required to snatch type I mRNA cap structures. The cap group is then used to prime the synthesis of viral mRNA (Blaas et al., 1982). PB2 interacts with PB1, and with the NP (Biswas et al., 1998) and contribute to viral pathogenicity, most likely by directly controlling levels of vRNA replication (Tscherne and García-Sastre, 2011). In addition to its transcriptional role, PB2 is also involved in replication because some mutations in PB2 have been shown to affect replication but not transcription (Gastaminza et al., 2003).
PB2 has also been shown to be associated with host range restriction of influenza and interspecies transmission barriers of AI to infect human (Subbarao et al., 1993). Numerous substitutions within the PB2 subunit have been shown to alter host range, virulence, pathogenicity, adaptation to mammalian hosts, and affect the transmissibility of IAV (Hatta et al., 2007; Massin et al., 2001; Steel et al., 2009a).

**Polymerase basic 1 (PB1)**

PB1 is the backbone of the viral RNA polymerase complex (Coloma et al., 2009). It is viral RNA dependent RNA polymerase responsible for the sequential addition of nucleotides during elongation of viral mRNA, viral complementary RNA (cRNA) and vRNA (Guu et al., 2008; Nakagawa et al., 1996) and contains the conserved motifs characteristic of RNA-dependent RNA polymerases (Chu et al., 2012). PB1 is also responsible for binding to the terminal ends of both vRNA (Li et al., 2001) and cRNA (González and Ortín, 1999) for initiation of transcription and replication. The interaction with the 3 prime end of the vRNA activates the endonuclease activity of PB1 (Cianci et al., 1995; Hagen et al., 1994; Li et al., 2001), which generates the capped primer required for mRNA synthesis. PB1 contains the nuclear localization signal for the polymerase complex to enter the cell nucleus and start genome replication (Asano and Ishihama, 1997; MacDonald et al., 2012).
Polymersase acidic (PA)

PA is the third subunit of the RNA polymerase complex. The function of PA is not very well understood, but PA has been shown to be essential for viral replication (Nakagawa et al., 1996). It was suggested that the N-terminal region of PA is involved in multiple functions of the polymerase, including protein stability, endonuclease activity, cap binding, and promoter binding (Hara et al., 2006). It has been proposed that PA binding to PB1 induces a structural change, which may enhance an interaction with cellular proteins resulting in nuclear translocation of the PA-PB1 complex (MacDonald et al., 2012).

Nonstructural viral proteins

1. NS1: host cell antagonist

The NS1 protein is designated as a non-structural protein because it is synthesized in virus infected cells, but is not present in virions. It is a multifunctional protein through its different domains, RNA binding domain and effector domain (Wang and Krug, 1996). The RNA binding domain is located in the N-terminal 73 aa, and is involved in the prevention of interferon induction and the interferon antiviral effects (Kochs et al., 2007), while the effector domain is the remaining 157 aa and carries out various functions through binding several host-cell proteins (Hale et al., 2008).

NS1 protein can inhibit host cell mRNA processing and blocks nuclear export of polyadenylated cellular transcripts by binding two host cellular protein, the 30 kDa
protein subunit of the cleavage and polyadenylation specificity factor and poly (A) binding protein II, through its effector domain (Lu et al., 1994; Nemeroff et al., 1998; Qiu and Krug, 1994). Additionally, NS1 has also been shown to interact with eukaryotic initiation factor 4G (eIF4G), associated with enhancing mRNA cap recognition, to promote viral translation through recruitment of eIF4F protein (Burgui et al., 2007). In combination with PA and PA-X endonuclease activities and the interaction of NS1 with eIF4Fs aids to decrease host cellular mRNA translation while promoting viral mRNA translation (Burgui et al., 2007; Crépin et al., 2010; Jagger et al., 2012). This trend of inhibiting host cellular translation while promoting viral mRNA translation also occurs through NS1’s interaction with U6 small nuclear RNA and splicesomal components. This interaction results in the recruitment of the spliceosome in the nucleus of infected cells inhibiting host cell mRNA splicing and promoting viral mRNA splicing, which is necessary for splicing of M1 and NS1 mRNA in order to translate M2 and NEP, respectively (Wang and Krug, 1998).

NS1 also plays a role in modulating the host immune response, by suppressing virus-induced host type I interferon response (García-Sastre et al., 1998). NS1 protein binds, sequesters double strand RNA through its RNA-binding domain (Donelan et al., 2003; Wang et al., 1999) and thus prevents activation of protein kinase R (PKR) and the 2′-5′ oligoadenylate synthetase/RNase L antiviral pathway (Bergmann et al., 2000; Min and Krug, 2006). Inhibition of PKR prevents the cellular stress response from down
regulating translation. Lastly, NS1 is associated with inhibition of caspase-1 dependent pathways which prevents maturation of proper interleukin responses and caspase-1 dependent apoptotic pathways (Stasakova et al., 2005).

2. **NEP**

The NEP, previously named NS2, is translated from a spliced NS1 mRNA and on the contrary of NS1 protein is incorporated into newly formed viral particles (Amorim and Digard, 2006). NEP is essential for the nuclear export of newly formed vRNPs, which are transported to assembly sites at the apical plasma membrane. NEP is able to bind to M1, which binds to vRNPs and CRM1, a host nuclear export protein, to allow for the export of newly formed vRNPs using the host's machinery (Akarsu et al., 2003; Neumann et al., 2000). NEP does not possess a NLS to aid in facilitating its function, but is believed to diffuse freely into the nucleus due to its small size, approximately 14.5 kDa (Neumann et al., 2000).

**Nonessential or accessory viral proteins**

The IAV genome encodes several proteins that are considered to be non-essential for replications, which include PB1-F2, PB1-N40, PA-X, PA-N155 and PA-N182 (Chen et al., 2001; Jagger et al., 2012; Wise et al., 2009). Three of those proteins (PB1-F2, PB1-N40 and PA-X) share the ability to alter the effect of viral infection in terms of replication, pathogenicity, and modulation of host cell signaling. Additionally, they all
share a property of being an alternate translation product of IAV polymerase mRNA, either from PB1 or PA through alternative translation pathway.

1. **PB1-F2: Pro-apoptotic membrane associated protein**

   PB1-F2 is found in a second open reading frame of the PB1 mRNA and thus named PB1-F2 (Chen et al., 2001). Since the initial report of PB1-F2, a number of functions have been attributed to PB1-F2 including induction of apoptosis, immunopathology, and secondary bacterial infection (Chen et al., 2001; Conenello et al., 2007; Varga et al., 2011; Weeks-Gorospe et al., 2012; Zamarin et al., 2005).

   A crucial role attributed to expression of PB1-F2 was the induction of the intrinsic apoptotic pathway: the induction of apoptosis through the permeabilization of the mitochondria and the release of cytochrome C (Chen et al., 2010; Zamarin et al., 2005). Additionally, modulation of both innate and cell-mediated immune response (CMI) is one of the known characteristics associated with PB1-F2 (Chen et al., 2001; Coleman, 2007; Varga et al., 2011). PB1-F2 antagonizes the intracellular interferon pathway through direct interaction with mitochondrial antiviral signaling protein, an essential mediator protein for transcriptional upregulation of IRF and NF-κB signaling pathways (Varga et al., 2011). It induces a pro-inflammatory CMI response through dysregulation of cytokines leading to infiltration of lung with neutrophil and severe pneumonia (Coleman, 2007), and this will promote or coincide with secondary bacterial infection and has been shown to increase the damaging effects of influenza and bacterial co-infection (Weeks-
Gorospe et al., 2012). The functions associated with PB1-F2 expression appear to be strain specific and full-length open reading frames for PB1-F2 are not always present in IAV isolates.

2. **PB1-N40: Viral transcriptional regulator**

PB1-N40 is the second translation product that results from a leaky ribosomal scanning of the PB1 mRNA initiated at 40 aa downstream of PB1 protein start codon. It shows interaction with PB2 and PA, but N40 does not possess polymerase activity and does not interact with vRNA and cRNA. Thus, it is primarily associated with maintaining balance between PB1 and PB1-F2 expression to maintain favorable replication levels. Previous report have shown that expression of PB1-F2 resulted in inhibition of PB1-N40 expression and delayed viral replication, but removal of both PB1-F2 and PB1-N40 resulted in no change. Therefore, N40 is not essential for viral replication, but provide a balance to maintain viral transcription in certain viral strains (Wise et al., 2009).

3. **PA-X: Host signaling antagonist**

PA-X is the most recently described IAV encoded protein and is predicted to be encoded in a majority of IAV isolates. PA-X is the resulting product of a frame shift from translation of the PA mRNA and possesses the N-terminal endonuclease portion of PA and a novel X-ORF aa coding sequence. Functionally, PA-X has been shown to repress cellular gene expression by specifically targeting host cell mRNA with its endonuclease activity. While the protein is considered to be non-essential because it is not necessary for
replication, loss of PA-X resulted in a stronger host response to IAV infection (Jagger et al., 2012).

**IAV replication cycle**

IAV replication can be divided into four main phases: (i) virus attachment and penetration into the host cell; (ii) transcription of the viral genome and translation of viral proteins; (iii) replication of the viral RNA; and (iv) assembly of the virions and subsequent release from the host cell. Initial exposure and infection with IAV occurs through either direct contact with an infected host or viral containing aerosolized droplets which enter the body through nasal or oral routes (Shaw and Palese, 2013). Upon entry into the host, IAV bind to host receptors through HA binding sites with different preferential sialic acid receptors (Couceiro et al., 1993). Once the viral HA has bound to the SA receptors, IAV entry takes place either via clathrin dependent or independent endocytic pathways leading to subsequent progression of the infection culminating in viral envelope and endosomal membrane fusion (Rust et al., 2004).

Once the virion is endocytosed into an endocytic vesicle, and the pH begins to drop, HA undergoes a low pH-induced conformational change that allows the HA2 subunit to imbed into the lipid membrane of the endosome, which then promote fusion of the viral membrane with the endosomal membrane by pulling the two closer (Skehel and Wiley, 2000). After envelope fusion, the capsid of M1 proteins is exposed to the cytoplasm, and M2 ion channel functions to allow escape of vRNPs into the cytoplasm.
Once in the cytoplasm, the vRNPs are transported to the nucleus through an interaction of NP with the importin α/β pathway. However, notably the viral proteins associated with vRNPs contain NLS that have been speculated to contribute to import (O’Neill et al., 1995; Wu et al., 2007b).

Once inside the nucleus, the vRNP and NP protein complexes catalyze transcription of vRNA into viral mRNA. Replication of the viral genome from viral mRNA template can occur later in infection, in which PA aids in the shift to produce full length cRNA which serves as template for negative strand vRNA (Perez and Donis, 2001; Watanabe et al., 2010). In order for influenza to generate mRNA, vRNP components must cleave 5’ caps from host pre-mRNA (Krug et al., 1979). The cap snatching serves at least two distinct purposes. First, this process allows the 10-13 nucleotides of the 5’ cap molecule to serve as a primer for transcription of negative strand vRNA, and second, the loss of the 5’ cap diminishes host pre-mRNA export and translation (Bouloy et al., 1978). The PB2 binds the 5’ caps of host pre-mRNA (Blaas et al., 1982), whereas the PA causes the endonuceolytic cleavage of the cap from the host pre-mRNA (Dias et al., 2009). Viral mRNA will be elongated, and polyadenylated through tracts of poly-uracil encoded toward the 3’ end of the mRNA template, which make the viral mRNA structurally indistinguishable from mature cellular mRNAs (Luo et al., 1991). In addition to cap snatching, influenza proteins directly target host mRNA to inhibit host cell transcription initiation and elongation (Katze et al., 1986). Influenza mRNAs in the cytoplasm are
preferentially translated, even prior to the depletion of cellular mRNA, which is believed to be due to the highly conserved 5'UTR sequence present on every IAV segment (Garfinkel and Katze, 1993). The 5'UTR of influenza mRNA contains Guanine rich sequence factor 1 (GRSF-1) binding recognition sequence 5'-AGGGU-3' (Gale et al., 2000). When the host encoded GRSF-1 binding protein bind to 5'UTR motif of influenza mRNAs, it will increase translation of viral proteins (Kash et al., 2002). After transcription, the viral mRNA is polyadenylated through a "stuttering" mechanism in which the viral polymerase encounters a poly-uracil region approximately 17 nucleotides from the 5’ end of the vRNA and transcribes back and forth over the poly-uracil region until the poly-(A) tail is completed (Poon et al., 1999). Viral mRNA products are then transported to either endoplasmic reticulum associated ribosomes (HA, NA, and M2) or cytoplasmic ribosomes (all other viral proteins) for translation.

The mechanism for the transition from viral protein translation to viral replication is still largely unknown. However, accumulation of either M1 or NP may be factors in the transition to replication (Martin and Helenius, 1991; Portela and Digard, 2002). It should be noted that transcription of viral mRNA, cRNA, and vRNA occurs throughout the viral life cycle, but transcriptional levels of the RNA molecules vary temporally (Kawakami et al., 2011). No matter the circumstance, initiation of replication results in the transcription of the intermediate cRNA and newly transcribed genomic vRNA through a self-priming secondary structure in the UTR sequence. Immediately after transcription, the newly
formed cRNA and vRNA molecules are encapsulated by NP to stabilize the RNA molecules and are bound to the tripartite polymerase to form new vRNPs (Klumpp et al., 1997). The NEP mediates efficient trafficking of vRNPs into the cytoplasm late in infection (O'Neill et al., 1998). Nuclear localized M1 then binds to vRNPs through NP and NEP, which in turn binds to CRM1 (a host nuclear export protein) resulting in the exportation of vRNPs to the cytoplasm. vRNPs then localize at the recycling endosome and the viral polymerase of the vRNPs then interacts with Rab11 (a host membrane trafficking protein) resulting in the transport of vRNPs to the apical plasma membrane (Akarsu et al., 2003; Neumann et al., 2000).

Late during infection, newly synthesized HA and NA proteins localize into lipid rafts on the infected cell surface, whereas M2 surface expression is more diffusely distributed. vRNPs then interact with the M1 scaffolding of virion assembly sites located at the apical plasma membrane to these HA/NA rich lipid rafts, initiating viral budding (Bron et al., 1993). Budding of IAV particles occurs through a cholesterol dependent manipulation of the membrane by an amphiphatic helical domain located in M2, providing a unique endosomal sorting complex required for transport independent mechanism for IAV budding (Rossman et al., 2010). Prior to or during release, the NA protein removes SA modifications from glycoproteins and glycolipids on the surface of the cell and virion, allowing for efficient viral release and preventing the formation of virion aggregates (Palese et al., 1974). IAV virions are then released into the respiratory
or intestinal lumen where they will go on to either infect other cells within the host or be released into the environment to infect a new host (Shaw and Palese, 2013).

1.3. **AI vaccines and vaccination strategies**

AI virus outbreaks caused by LPAI and HPAI viruses have inflicted economic loss to the U.S. and world poultry industry. Despite control efforts, the disease continues to spread, causing economic losses, threatening the livelihood of hundreds of thousands of struggling poultry farmers, and seriously impeding regional and international trade and market opportunities. IAV continuously change by antigenic drift and reassortment resulting in antigenic shift which make the development of an effective vaccine a challenge for scientists (Tosh and Poland, 2008).

IAV use several strategies to evade host immune system and immune defenses. Under the host immune pressure minor point mutations occur gradually in the viral genome. Error-prone RNA-dependent RNA polymerases are the main cause of these minor, but frequent, point mutations which result in changes of the viral surface protein. This is known as antigenic drift and may result in the loss of effectiveness of existing antibodies in the host (Suarez and Senne, 2000). Host cells that are co-infected with two or more IAV may result in gene reassortment with a generation of a new virus composed of gene segments from two different viruses, a process known as antigenic shift (Zambon, 1999). Because of the high propensity of IAV for mutation, vaccines must continually be
updated to keep pace with the variation in surface antigens and combat newly generated viruses.

Vaccination remains a legitimate tool in supporting eradication programs especially if used in conjunctions with other AI control methods. Vaccines and vaccination have been shown to increase the resistance of poultry to infection, reduces virus shedding if vaccinated birds are infected, and reduces the transmission dynamics (Capua and Marangon, 2006). However, the possible drawbacks of vaccination are inability to produce sterilizing immunity with appearance of new strains, ongoing cost of vaccination program, and inability to control and ultimately eradicating the infection when used alone (Sims, 2012). Thus, vaccination programs must be part of a comprehensive strategy with other control components of stamping-out programs to achieve proper control and eradication of AI (Swayne et al., 2014).

An ideal vaccine must be safe, effective, shelf-stable, inexpensive, cross-subtype specific, easily administered, and protective with minimal inoculations (Beverley, 2002; Levine and Sztein, 2004). In addition, it should also enable differentiation between infected and vaccinated animals (DIVA) (Peyre et al., 2009). According to APHIS of the USDA, several criteria should be considered in a newly designed vaccine before approval for use in animals that include; purity (exclusively desired compounds, consistent in production), safety (no harmful effects on the host or environment), efficacy (quantified
standards of protection), and potency (protection in a variety of conditions, reasonable dosages) (Rao et al., 2009; Swayne, 2009).

Numerous studies have demonstrated that vaccines against AI under experimental conditions can confer adequate protection from disease and prevent transmission of virus (Sims, 2013). Four different categories of vaccine technologies, inactivated whole virus, live attenuated, gene based (recombinant, subunit, and DNA), and virus like particles vaccines, have been used to develop AI vaccines in the laboratory and study their ability to protect birds. However, application in the field through licensing and use has only been accomplished with a few technologies and products: i.e., inactivated whole virus and live vectored vaccines (Swayne and Spackman, 2013). Here, we briefly review the different types of vaccines followed by field vaccination strategies.

1. **Inactivated influenza vaccines (IIV)**

Conventional IIV are the major type of vaccines used in the poultry industry. Conventional AI vaccines are produced with whole AI virus of a specific subtype grown in embryonating chicken eggs (infectious allantoic fluid). The harvested infectious fluid containing live virus is inactivated by a series of chemical and physical processes (Cinatl et al., 2007; Marangon et al., 2008; Qiao et al., 2006), making them harmless to the host as the virus cannot replicate. These vaccines, either as monovalent or polyvalent, elicit significant immune response in a variety of poultry species especially when administered
with an adjuvant. Multiple doses of the vaccine are needed to achieve a desired protective antibody titer and this is the major drawback of these vaccines.

The invention and application of reverse genetics (RG) technology has revolutionized the vaccine industry. Use of this technique provided the opportunity to prepare custom-made vaccines specifically for HPAI viruses, converting them to LPAI by removal of multiple basic aa at the HA cleavage site (Horimoto and Kawaoka, 2006; Marsh and Tannock, 2005). This method is more efficient, precise, and versatile than developing a seed strain via the traditional method of attenuation, which requires multiple passages of a virus in eggs until the virus loses virulence. Such vaccines have been evaluated for immunogenicity and efficacy against AI in poultry demonstrating similar protection and performance to conventional IIV (Tian et al., 2005; van den Berg et al., 2008; Webster et al., 2006b).

Adjuvant is used in vaccine to induce the desired immune response and for a prolonged high titer antibody production (Cox et al., 2004). Most common adjuvants used in poultry is oil in water emulsion. The oil phase functions as an inert depot resulting in prolonged antigen residence and slow release of emulsified antigen. Induction of a local inflammatory response at the site of the injection is also an important factor in attraction and stimulation of inflammatory and antigen presenting cells. This in addition to a prolonged and continuous release of emulsified antigen will result in stimulation and boosting of the immune response (van den Berg et al., 2008).
IIV against AI are either homologous or heterologous, depending on the choice of the viral strain. The homologous vaccine is prepared from the same HA and NA subtypes of the circulating field virus. This system will detect the field exposure by testing unvaccinated sentinels left in the flock which might be discouraging in premises that contain floor-raised birds. Determination of anti-NS1 antibodies with this type of IIV will allow detection of field exposure of vaccinated birds (Tumpey et al., 2005). Alternatively, the heterologous or marker vaccine contains the HA subtype of the circulating wild virus but a different NA subtype will facilitate the DIVA strategy (Sims, 2007). The antibodies to HA will provide cross-protection, increased resistance to challenge and reduction of shedding, while antibodies to the NA of the field virus can be used as a natural marker of infection in vaccinated birds. This system had been used to support eradication programs against several introductions of LPAI viruses (Capua et al., 2004).

It is important to note that in poultry, variable quantities of virus shedding may occur, even in birds that have received vaccine and are considered protected. The vaccine immune efficacy will be influenced by the antigenic relatedness of the vaccine and the challenge strains, the amount of HA antigen, and the vaccination schedule. High antigenic relatedness between the vaccine and the field strains is the most critical for optimal immune protection against challenge virus (Tian et al., 2010). Thus, it is essential
to conduct surveillance and antigenic analysis of field strains to allow timely update of the vaccine.

The production of an IIV usually takes 6-8 months; thus, in response to the emergence of a severe AI outbreak the vaccine may not be available in time to stop and control the spread. In addition to the time limitation, HPAI viruses may kill the embryo rapidly in the production phase which makes direct propagation of vaccine candidate virus difficult (Wright, 2008). Moreover, the parental injection of IIV to every individual bird in the flock can be cumbersome, expensive, and time-consuming in commercial poultry flocks.

2. **Live attenuated influenza vaccines (LAIV)**

Developing LAIV is gaining an increased interest as it mimic the natural infection. IAV antigens are presented to the host immune system via different pathways, exogenous or endogenous, leading to induction of cross-reactive cellular response, humoral antibody response as well as mucosal immunity especially after intranasal administration (Cox et al., 2004; Watanabe et al., 2009). Using RG techniques, LAIV acquires multiple mutations in its viral genes that produce the cold-adapted, temperature-sensitive and attenuated phenotypes. The cold-adapted vaccine viruses could replicate and grow only when temperature is below 25 °C and stop growth when temperature exceeds 37.8 °C (Esposito et al., 2012). The temperature-sensitive phenotypes means that viral replication is highly efficient at 33 °C, but becomes ineffective as temperatures reaches 39 °C
The attenuated phenotypes refers to a vaccine created by reducing the virulence of a pathogen, but still keeping it viable, for instance through truncation of NS1 protein and mutation of PB2 protein (Steel et al., 2009b).

LAIV has been developed for intranasal use in humans (FluMist®) and equines (Belshe et al., 1998; Davenport et al., 1977; Paillot et al., 2006). In poultry, Nang et al. (2013) used RG to produce a cold adapted H5N1 clade 1 (A/VietNam/1203/2004) virus after introducing mutation in the HA proteolytic cleavage site to a LPAI virus sequence in a backbone of an H9N2 LPAI and demonstrated protection against mortality with homologous challenge which was improved after two vaccinations versus one. IAV with truncated NS1 gene were rescued using RG technique and tested as LAIV candidates. Wang et al. (2008) reported that A/turkey/Oregon/71-delNS1 (H7N3) virus (10 nucleotide deletion in the coding region of the NS1 gene) could be used as a potential LAIV. The NS1-truncated H7N3 LAIV viruses were highly attenuated in chickens and did not transmit the virus from infected chickens to un-inoculated cage mates. At the same time, the candidate viruses induced relatively high antibody titers, which conferred good protection against a high dose heterologous virus challenge (Wang et al., 2008b). Moreover, use of LAIV with truncated NS1 gene in swine and equine showed a good level of protection (Quinlivan et al., 2005; Richt et al., 2006; Vincent et al., 2007).

LAIV offer several advantages over IIV: they can be mass applied, provide reasonable immunity after a single dose of vaccination, induce cross-reactive immune
responses and trigger mucosal and cellular immunity (Heinen et al., 2002). Many studies have demonstrated that intranasal immunization with LAIV could not only elicit IAV-specific secretory IgA antibodies and serum IgG antibodies, as well as T-cell responses, but also could provide cross-protection against heterologous IAV (Belshe et al., 2004). LAIV also induces a robust memory response, including the production of chemokines and cytokines involved in T-cell activation and recruitment, which can then clear the virus rapidly (Lanthier et al., 2011). However, the World Organization for Animal Health (OIE) and Food and Agriculture Organization do not recommend the use of LAIV in poultry because of risk of adaptation of H5 and H7 vaccine strains to chickens and/or reassortment with circulating field IAV which may potentially generate a new HPAI virus. In addition, DIVA strategy may be challenging in vaccinated flock with LAIV (van den Berg et al., 2008).

3. Gene based (Subunit, Recombinant and DNA) vaccines

As mentioned earlier, RG technology represents a substantial improvement in generating inactivated and live attenuated vaccine prototype strains. RG enables the selective modification of different viral genes and thus formation of custom made vaccine seed strains. RG bridges the concept of traditional vaccines and modern gene based vaccine (recombinant, subunit and DNA vaccines). Gene based vaccines differ from conventional vaccines in their ability to allow selective expression and delivery of immunogenic viral gene products in vaccinated rather than injecting preformed virions or
viral proteins, as done conventionally. Thus, these vaccines are designed to be free of contaminants and to maximize safety and immunogenicity; as virulence genes can be removed to mitigate any risks posed by reassortment with field strains (Rao et al., 2009). Those vaccines offer another advantage as they can be custom-made to target specific field strains or emerging threats in a short production time and on a large scale (Swayne, 2009).

*In vitro* expression of IAV gene(s) is applied to design subunit vaccines. A subunit vaccine is an immunizing agent that contains viral proteins, but no viral nucleic acid and therefore induces a focused immune response (Rahn et al., 2015). It comprises of at least one recombinant IAV protein, which in most cases is the HA, which can be expressed in baculovirus expression system and the protein can be later used as vaccines along with adjuvants (Kuroda et al., 1986). A baculovirus expressing AI HA protein (H5 or H7) completely protected vaccinated chickens against challenge with the homologous virus and abolished or reduced viral shedding (Crawford et al., 1999). In another study, vaccination of chicken and mice with HA of an H5N1 HPAI and were able to induce a strong antibody response and afford full protection from homologous lethal challenge (Liu et al., 2013).

*In vivo* expression of IAV genes using a genetically engineered and modified vector (virus or bacteria) is another strategy for introducing immunogen to the host (Minke et al., 2006; Poulet et al., 2007; Stittelaar et al., 2010). Recombinant vectored
vaccines offer several advantages over IIV for oral or mucosal administration because of the flexibility in the choice of a vector suitable for these administration routes: good immune response to a replicating virus (no adjuvant and less starting antigen needed), some vectors may be mass applied and both costs and efficiency are improved by giving two vaccinations with the labor required for one. To date, a variety of recombinant vaccines for AI virus have been developed by using fowl pox virus (FPV), herpesvirus of turkeys (HVT), Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV), and duck enteritis virus (DEV) (Liljebjelke et al., 2010; Spackman and Swayne, 2013). In particular, FPV recombinant vaccines expressing H5 or H7 of AI subtypes and NDV have been licensed for use in the field in Central America (FPV-vectored vaccine), Southeast Asia and Egypt (NDV-vectored vaccine) (Rahn et al., 2015). Each type of those recombinant vaccines has its pros and cons. A specific advantage of HVT vectored vaccines is that they can be given in ovo which saves labor and reduces interference from maternal antibodies, although the duration of immunity is not clear. Moreover, Potential disadvantage which has been observed with FPV vaccines is that they may not confer adequate immunity if the bird has been exposed to or vaccinated with the vector previously (Swayne et al., 2000). The application of the recombinant ILTV vaccine is limited because it is only needed in older layers in the few areas where ILTV is circulating. Therefore, the bivalent ILTV vectored vaccine is of limited significance in the control of AIV compared with other live virus vector vaccines.
DNA-based vaccines are based on plasmids expressing the encoded protein and are used for the transfection of cells, resulting in the induction of both cellular and humoral immune responses (Liu, 2011). Different improvements of this technique have been made, especially regarding the stability, expression, delivery, and immunogenicity since its first description in mice (Rahn et al., 2015). On experimental studies it has been found that chickens given a single dose of plasmids expressing H5 and H7 HA were protected from infection by either subtype (Kodihalli et al., 2000). Additionally, vaccination of chickens with different plasmid DNA encoding the HA, NA and NP genes from an AI in combination with IL-15 and IL-18 induced high antibody titers and CMI leading to the survival of challenged birds (Lim et al., 2012) A significant advantage of DNA vaccines is their ability to encode multiple genes of interest (Chen et al., 2012), plus they can also be used for preparation of reference antisera as it allows the production of HA specific antibodies without producing antibodies specific to other IAV proteins (Lee et al., 2003). Nevertheless, big concerns for the use of DNA vaccines are the danger of genomic integration into the host cell and the presence of selective markers, such as antibiotic resistance genes (Wang et al., 2004).

4. Virus-like particles (VLPs)

The expression and self-assembling of viral structural proteins into VLPs represents another promising type of vaccine (Latham and Galarza, 2001). VLPs are multimeric, sometimes multiprotein nanostructures that are assembled from viral
VLPs contain repetitive high-density displays of viral surface proteins and thus they are a highly adaptable platform for various applications. Importantly, they contain functional viral proteins responsible for cell penetration by the virus, which ensures efficient cell entry. In other words, they resemble infectious virus particles in structure and morphology and induce a sufficient immune response but do not contain any viral genetic material and are therefore not infectious.

Several systems for the production of influenza VLPs have been described, including baculovirus, transient plasmid expression, stable cell-line transformation and expression in plants, but mostly mice or ferrets were used for vaccine studies. In chickens, a baculovirus expression system was used to develop VLPs with HA and M1 from a H9N2 virus. Vaccinated birds elicited high levels of antibody responses and reduced viral shedding (Lee et al., 2011). In similar approaches, other groups generated VLPs comprising HA, NA and/or M1 from a H5N1 HPAI virus and showed that immunized chickens were protected against a lethal homologous challenge without development of clinical signs of infection (Park et al., 2013).

5. Vaccination strategies

A basic rule to follow when implementing vaccination against AI is that the use of vaccines is only one of several tools to prevent or contain an outbreak spreading in
unaffected flocks (Peyre et al., 2009). Thus, three different vaccination strategies could be applied according to the infectivity level of the country (Capua and Marangon, 2007).

Preventive vaccination could be used in AI free country free of disease but at high risk of introduction of the disease. All birds at high risk should be vaccinated. This strategy suggests a major cost-benefit issue in developing countries where vaccination against other poultry circulating diseases such as Newcastle disease is not properly performed.

Emergency vaccination should be conducted during an outbreak. All unaffected animals within and around the outbreak quarantine zone should be vaccinated. Vaccination zone, determined within the contingency control plan, depends on the transmission rate and initial AI spread during the high-risk period. Preventive vaccination should be used in synchronization with emergency vaccination within a country if the risk of virus spread is high.

Prophylactic vaccination should be applied when the disease has become endemic. Birds are vaccinated systematically against the same HA subtype of the circulating filed virus to attain protection level in the vaccinated flocks. The long term goal of this approach is control and eradication of the disease within the country on all commercial and backyard poultry. Once the disease is controlled, biosecurity measures and stamping-out can achieve eradication. This strategy also implies major practical and cost-benefit issues for implementation by developing countries.
1.4. Matrix protein 2 as a vaccine candidate

The hallmark of IAV is the remarkable variability of its major surface glycoproteins, HA and NA, which allows the virus to evade existing anti-influenza immunity in the target population. Thus, the conventional influenza vaccine must be adapted almost every year to follow the new changes of the virus. In these circumstances, a broad-spectrum vaccine against IAV is mandatory. M2 protein, the third membrane protein of IAV, is a promising target to develop a broad-spectrum vaccine. Previous reports have shown the ability of anti-M2 antibodies to confer protection against IAV infection. Herein, a more comprehensive description of M2 protein is provided, since this protein is the antigenic target of the vaccine examined in this thesis.

Structure and domains

M2 is a 97 aa type III transmembrane protein (Iwatsuki-Horimoto et al., 2006; Sakaguchi, 1997). M2 is a homo-tetramer with disulfide-linked M2 dimers as a basic unit which holds the final tetrameric form through non-covalent and hydrostatic interactions (Holsinger and Lamb, 1991; Sugrue and Hay, 1991). M2 protein is abundantly expressed at the plasma membrane of infected cells but only a small number of molecules (16-20 / virion) are incorporated into virions compared with ~500 HA molecules/ virion (Schroeder et al., 2005).
M2 protein can be divided into 3 major domains: a 24 aa extracellular N terminal domain (M2e, residues 2-24), a 19 aa transmembrane domain (TM, residues 25-46) and a long 54 aa intracellular C terminal domain or cytoplasmic tail (residues 47-97).

1. **The transmembrane domain (TM)**

   The TM domain (residues 25 - 46) forms a four-helix bundle which drives the tetramerization and forms the pore of the low-external-pH-sensitive ion channel. Crystal structure analysis revealed that the TM domain acts as a proton transporter with valine at position 27 and histidine at position 37 functioning as a gate (Khurana et al., 2009). Sharma et al., (2010) proposed a mechanism for the selective proton channel function of M2. In their model, histidine at position 37 and tryptophan at position 41 face the inside of the tetrameric TM channel and guide protons by forming and breaking hydrogen bonds between adjacent pairs of histidine and through interactions of the histidine with the tryptophan gate. Moreover, this TM acts as an internal transmembrane anchor signal which was evidenced by absence of a cleavable transmembrane insertion signal (Finidori et al., 1987). TM also contains a cholesterol-binding region and has been shown to localize some of the surface M2 to the lipid raft region where HA and NA are densely aggregated (Schnell and Chou, 2008).

2. **The cytoplasmic tail (CT)**

   The CT plays an important role in stability of M2 tetrameric structure. Additionally, it aid in virion assembly, coordinating with HA, NA and M1 proteins for
successful viral genome packaging, budding and virus production (Iwatsuki-Horimoto et al., 2006). Moreover, Rossman et al., (2010a) have shown that CT removal would result in failure of vRNPs inclusion in newly formed virions with further attenuation of IAV and thus affecting virus budding and release from cells.

3. **The Extracellular domain (M2e)**

The M2e, 24 aa, has been targeted as IAV vaccine candidate due to the high sequence conservation between different IAV (Neirynck et al., 1999; Oxford, 2013). Such strong sequence conservation is remarkable for an RNA virus that exists in many subtypes and strains, and it is in sharp contrast with the variability of HA and NA (Sharp, 2002). In the latter case, nearly any aa change is tolerated, whereas in the case of M2e, selection pressure is also imposed by the overlapping M1 codon sequence.

A few explanations have been proposed for the limited variation in M2e sequence:

a. The genetic relation between M2e and M1. M2 is expressed from a spliced mRNA derived from the M1 mRNA (Lamb and Choppin, 1981). M1 and M2 share the first 9 aa at their amino termini. Additionally, residues 10–23 of M2e and 239–252 of M1 are also encoded by the same RNA sequence but are translated by different reading frames (Lamb et al., 1981). Comparison of those two regions will reveal that first 9 aa residues are highly conserved even in the new bat isolates. While residues 10-24 are more variable which might be related to the species specificity of those sequence. Liu et al., (2005) has compared and aligned 716 IAV M2e
sequences available in GenBank. The analysis have revealed that one region (aa 10–20) have higher variability than other regions on M2e protein which was further categorized according to either HA subtype or host restriction specificities. The aa change in this region was consistent with host restriction specificities of human, avian and swine species: PIRNEWGCRECN (human), PTRNGWECKCS (avian) and PIRNGWECRCN (swine). In other aspects, over 85.4% of human influenza M2e proteins contained isoleucine (I) at aa 11, while over 90% of AI M2e proteins contained threonine (T) at aa 11. In contrast, T (57.5%) and I (42.5%) occurred at a similar frequency in swine M2e protein at aa 11. For aa 14–16 on M2e protein, avian and swine IAV have the same most common sequence, GWE, while the most common sequence of human IAV was EWG. For aa 18–20, human and swine IAV shared the same most common sequence, RCN, while the most common sequence of AI was KCS. Interestingly, these comparison results indicate that the human IAV might be more closely related to the swine IAV than to the AI virus, which was also consistent with previous studies by evolutionary analysis of the HA gene of IAV (Fouchier et al., 2004; Webster et al., 1992). Additionally, some residues as arginine at aa 12, tryptophan at aa 15, cysteine at aa 17, cysteine at aa 19, and serine at aa 22 are strongly conserved suggesting that these residues are functionally important (Deng et al., 2015).
Following infection or vaccination with inactivated, licensed influenza vaccines, serum M2e specific responses are either weak or transient as evidenced by the low antibody levels in humans, ferrets, pigs and mouse (Black et al., 1993; Feng et al., 2006; Mozdzanowska et al., 2003). The adaptive immune responses directed to M2e are hardly induced as noted by post-infection antibodies to M2 are rare and that only 1–2% of persons in the community have antibodies to M2e (Feng et al., 2006). This could be attributed to the shielding effects by HA and NA proteins which might prevent the access of immune effector cells to M2e protein. Hence, there is probably only a low natural immune pressure directed against M2e.

**M2 function**

M2 is a multifunctional membrane protein acting as a viroporin or ion channel (Schnell and Chou, 2008). Their main functions are as follows:

1. M2 ion channel protein plays a vital role during viral uncoating and genomic entry into host cytoplasm. Soon after virion entry in the host cell, M2 forms an ion channel that is activated by the endocytic pH drop in the virus-containing vesicle, simultaneously with HA membrane fusion (Helenius, 1992). The M2 proton channel, at neutral pH 7.0, resides in its closed conformation as a tryptophan gate which is formed through molecular interactions between a tryptophan and aspartic acid (Schnell and Chou, 2008). As a result of the acidic pH, M2 conducts the flux of H⁺ ions across the viral membrane into the virion interior, protonation of
histidine residues and disruption of the tryptophan gate causes electrostatic repulsion, relaxing the channel opening to allow water to conduct protons to the interior of the virion (Schnell and Chou, 2008; Schweighofer and Pohorille, 2000). This proton influx loosens the interactions between the vRNPs and M1, a process that is named “priming”. M2 also allows the influx of potassium ions (K⁺) and sodium ions (Na⁺) (Leiding et al., 2010). The influx of K⁺ into the virion interior causes a second priming event during which the conformation of M1 is changed further and the vRNPs become relaxed and eventually the electrostatic interaction between M1 and vRNPs are lost (Stauffer et al., 2014). The low endosomal pH also triggers the membrane fusion activity of HA, which catalyzes the fusion of the viral envelope with the endosomal membrane and accompanied by the release of the vRNPs into the cytosol (Helenius, 1992). Thus, vRNPs dissociation from M1 allows escape of genomic, replicationally competent vRNPs into the cytoplasm. Once in the cytoplasm, the NP protein component of the vRNP complex, reveals a NLS and recruits cellular components to shuttle the vRNP complexes to the nucleus (Watanabe et al., 2010).

2. M2 ion channel protein is vital to pH regulation during late influenza infection. M2 is ubiquitously expressed throughout the trans-Golgi network (TGN) and by its proton transport actively increasing the pH of the TGN to equal that of the cell's cytosol rendering the Golgi less acidic (Ciampor et al., 1992a; Ciampor et al.,
This neutralization of TGN's low pH is necessary in order to prevent the HA protein conversion to its low pH-induced fusogenic form. This would render HA incapable of causing membrane fusion, halting future infectious cycles as this means that HA could convert to the post fusion state while in transit to the cell surface. Therefore, M2 pH regulation is vital to maintain HA in its neutral-pH, pre-fusion conformation. Additionally, the M2 proteins of different viruses vary in their ability to alter the trans-Golgi pH and the proton gating activity of M2 seems to have co-evolved with the differences in the pH that triggers membrane fusion of HA in these viruses (Betakova et al., 2005; Grambas and Hay, 1992).

3. M2 ion channel activity could influence on innate host immune response. The ion channel activity of M2 can trigger pro-inflammatory host responses by activating NLRP3 inflammasomes, through 2 different signals, in IAV infected cells (Ichinohe et al., 2010). This activation requires M2 ion channel activity at the Golgi apparatus and operates in macrophages and dendritic cells (DC). More specifically, IAV infection activates signal 1 through stimulation of macrophages and DCs via TLR7, which leads to synthesis of pro-IL-1β and pro-IL-18. After infection, virus-encoded M2 is expressed in the secretory compartment, including the TGN. The ion-channel activity of M2 enables the export of $H^+$ from acidified Golgi, and such activity is a trigger for signal 2, which is required for formation of the NLRP3
inflammasome complex. In addition to imbalances in the concentration of H⁺, imbalances in the concentrations of other cations (Na⁺ and K⁺) can signal inflammasomes activation. This finding suggested that ionic perturbation in the TGN by M2 channel activity triggers inflammasome activation. Moreover, M2-dependent IL-1β production by IAV-infected bone marrow-derived macrophages can be blocked by high concentration of extracellular K⁺ or by adding reactive oxygen species inhibitor. How M2 affects ROS production and K⁺ efflux remains unclear (Pang and Iwasaki, 2011). Viral infection and activity of the M2 channel was needed for full triggering of NLRP3 inflammasome activation which collectively suggest that sensing of cellular stress imposed by imbalances in ionic concentrations in intracellular vesicles could serve as a pathogen-recognition pathway.

M2e as vaccine candidate

1. General overview of M2e-based vaccines

The M2e protein have been considered an attractive antigenic target for developing a universal influenza vaccine. As mentioned in earlier section, the N-terminal epitope (residues 1-9) in M2e was found to be 100% conserved among different IAV strains. The remaining residues (10-20) show few amino acid changes depending on the host species (human, avian, swine, equine and other hosts) where IAV were isolated (Fiers et al., 2004; Liu et al., 2005).
Apart from its highly conserved sequence, M2e protein as 24 aa residue is a small peptide and a very weak immunogen in its native form. This was evidenced by low prevalence of anti-M2e antibodies in sera of infected patients (Black et al., 1993). Moreover, infection of mice with IAV did not induce significant levels of antibodies recognizing native M2 (Jegerlehner et al., 2004). *In vitro*, M2e-specific monoclonal antibody (14C2) have proved the role and function of M2 during IAV infection as evidenced by their ability to reduce either the plaque size or growth of some influenza strains in a strain specific manner (Hughey et al., 1995; Roberts et al., 1998). Additionally, passive transfer 14C2 monoclonal antibodies reduced the lung viral titers in mice (Treanor et al., 1990; Wang et al., 2008a). Aforementioned studies have shown the evidence that anti-M2 immunity can confer protection against different IAV and induction of adaptive M2 immunity would be an effective strategy for controlling IAV epidemics and pandemics.

Extensive research has been performed to enhance the immunogenicity of M2e in order to be used as a vaccine antigen and conferring protection against IAV infection. For that purpose different approaches to link M2e to carriers and/or use of potent adjuvants were explored. One of the earliest studies, a partially purified M2 protein, expressed from baculovirus infected cells, combined with incomplete Freund's adjuvant was used for vaccination of mice. The vaccinated mice showed a higher survival rate compared to non-vaccinated group after challenge with IAV plus induction of serum antibodies reactive
with synthetic peptides defining 3 antigenic determinants located on both the external N and internal C termini of M2 protein (Slepushkin et al., 1995). Later, several studies have been dedicated to develop different M2e fusion constructs using different carriers or systems: hepatitis B virus core (HBc) particles (Fan et al., 2004; Neirynck et al., 1999), human papillomavirus L protein VLPs (Ionescu et al., 2006), phage Q[beta]-derived VLPs (Bessa et al., 2008), keyhole limpet hemocyanin (Tompkins et al., 2007), bacterial outer membrane complex (Fu et al., 2009b), liposomes (Ernst et al., 2006), flagellin (Huleatt et al., 2008), and much more as summarized in Table 1.1.

One of the earliest candidate vaccines used in mice is 240 copies of M2e presented on the surface of the HBc capsid, which was highly immunogenic especially in the presence of adjuvant. Moreover, M2e-specific antibodies conferred full protection against a lethal IAV infection. This vaccine candidate led our current understanding of M2e vaccine protection. Sera from mice immunized with M2e-HBc vaccine was shown to passively protect mice from lethal intranasal IAV challenge. Such protection was lost in mice depleted of natural killer (NK) cells which supported antibody dependent cell mediated cytotoxicity (ADCC) as the potential mechanism of protection. Additionally, the protection was maintained in both complement-deficient and T cell deficient mice excluded the contributions of these immune pathways toward protection (Jegerlehner et al., 2004). In another study, vaccination of FcγRI and FcγRIII-deficient mice with M2e-HBc reveled the role of Fc receptors in M2e-specific ADCC protection. Additionally,
passively transferred wild type alveolar macrophages, in addition to NK cells, were capable of rescuing FcγRI and FcγRIII-deficient mice from lethal influenza infection (El Bakkouri et al., 2011). These two studies have shown that the protective mechanisms in mice requires cooperation of both humoral and innate immune responses.

Several M2e vaccines have provoked high levels of anti-M2e antibody responses. The animal models summarized in Table 1.1 reveal that M2e can be immunogenic in several vaccine constructs with a range of adjuvant and vectors. All candidate M2e vaccines generated significant increase in IgG (all 4 subclasses), IgA, with only one vaccine inducing detectable IgM (De Filette et al., 2008a; Denis et al., 2008; Fan et al., 2004; Huleatt et al., 2008; Jegerlehner et al., 2004; Liu et al., 2003; Liu et al., 2004b; Neirynck et al., 1999; Okuda et al., 2001; Schotsaert et al., 2009; Slepushkin et al., 1995; Talbot et al., 2010; Wolf et al., 2011; Wu et al., 2007a). Although T cell involvement in M2e-vaccine protection was not directly analyzed with every vaccine, isotype switching to IgG and/or IgA antibodies occurred with each vaccine as these were naive animals with no previous exposure to M2e antigen. M2e-specific B cells must have undergone isotype switching in a T cell dependent or independent fashion (Jegerlehner et al., 2004).

Free synthetic M2e-peptide with different adjuvant is the simplest M2e-vaccine design shown to elicit M2e-specific IgG response in mice and chickens that resulted in protection against IAV challenge. Moreover, use of different kinds of platforms or vaccination with M2e recombinant proteins in combination with adjuvant revealed
induction of significant antibody response against M2e. The level of antibodies and their different subclass distribution appear to be affected by the carrier proteins. The carrier proteins used in vaccine designs utilizes a unique manufacturing process, from completely synthetic to recombinant protein expression in bacterial or mammalian cell culture and purified, but they all have one thing in common: conversion of non-immunogenic M2e to highly immunogenic M2e to confer protection against different IAV.

Another approaches have been developed in order to overcome the linear presentation of M2e antigens on the carrier and in a particulate form, which enables a strong immune response as well as increasing their stability and immunogenicity. Additionally, M2e-mediated protection was relatively weak or partial compared with HA-mediated protection, plus M2e-specific antibodies were not very effective in binding to the virus (Jegerlehner et al., 2004). This is probably due to chemical or genetic fusion of M2e would not form the tetrameric structure of M2 in its native conformation. Researchers have pursued different techniques to facilitate the formation and maintenance of tetrameric structure for improving the immunogenicity of M2e and formation of antibodies recognizing the natural tetrameric M2e form. For instance, linking M2e to a tetramer forming leucine zipper domain of the yeast transcription factor GCN4 resulted in production of recombinant tetrameric M2e vaccines (De Filette et al., 2008a). The vaccine induced potent M2e-specific antibody responses and 100% survival.
protection to the vaccinated mice. Similar to that approach is presenting a full-length M2 on influenza M1-VLP without HA and NA using the baculovirus insect-cell expression system forming M2-VLP (Song et al., 2011b). As expression of M2 proteins in a membrane-anchored form mimicking the native conformation on influenza virions is critical for inducing effective protection by M2e-based vaccines. Mice vaccinated with M2-VLP vaccines induced high levels of M2e-specific antibodies compared with IIV. In addition, M2e-specific antibodies induced by M2-VLP were highly cross-reactive to different IAV subtypes (H1N1, H3N2 and H5N1) (Song et al., 2011a; Song et al., 2011b). Another group of researchers have used this technique to present multiple copies of M2e proteins on VLP (M2e-5x VLP). Immunization of mice with M2e-5x VLP induced protective antibodies that were cross-reactive to different IAV plus conferring cross protection in vaccinated mice. Anti-M2e antibodies induced by M2e-5x VLP showed a wider range of cross reactivity to IAV than those by M2e VLPs, or M2e monoclonal antibody 14C2 (Kim et al., 2013b; Lee et al., 2014a). Therefore, M2 proteins presented in a membrane anchored form on VLPs can be a promising approach to avoid the immune-dominant HA proteins and are effective in inducing M2 antibodies reactive to antigenically different influenza virions.

M2 is a structural protein that is abundantly expressed on the surface of infected cells (Lamb et al., 1985) in tetrameric form (Holsinger and Lamb, 1991; Sugrue and Hay, 1991). In other words, it is exposed to the adaptive immune system of the host.
Nevertheless, IAV infection induces a weak anti-M2 antibody response which is due to the shielding effect of the other surface glycoproteins (HA and NA) (Gabriel et al., 2008; Perez and Donis, 2001), and low M2e expression level on the virion surface (Zebedee and Lamb, 1988). Additionally, experimental infection of chickens with AI results in a modest but detectable anti-M2e specific serum antibodies. This finding has practical implication with the DIVA strategy (Kochs et al., 2007; Li et al., 2006). Ducks, for example, that were inoculated with IIV developed robust HI titers but undetectable M2e specific antibodies (Kochs et al., 2007). From these experiments, one can conclude that M2e-specific antibody are poorly induced in the unprimed host, but that primary infection elicits a degree of B cell immune memory against M2e that can be boosted by a subsequent heterosubtypic viral challenge. To summarize, following natural or experimental infection with IAV, M2e-specific antibody responses in circulation remain low. Therefore, pre-existing anti-M2e immune responses are unlikely to interfere with M2e-based vaccines.

Among those different vaccine platforms, investigators detected an M2e-specific CD4\(^+\) or CD8\(^+\) T cell response (El Bakkouri et al., 2011; Eliasson et al., 2008; Kim et al., 2013b; Okuda et al., 2001; Tompkins et al., 2007; Wu et al., 2007a). CD4\(^+\) T cell activation was measured through ELISPOT (IL-4 and/or IFN\(\gamma\)), ELISA (IFN\(\gamma\)), and cell proliferation assays with M2e peptide stimulated lymphocytes. Additionally, passive transfer studies has been performed after depletion of CD4\(^+\) and/or CD8\(^+\) and T cell
response was abolished. Only one study has been performed in chicken where birds was vaccinated with a recombinant M2e protein fused to HSP70 (Dabaghian et al., 2014). Birds showed a cellular immune response characterized by increased levels of Th1-type (IFN-g) and Th2-type (IL-4) cytokines production and increased CD4+ to CD8+ ratios.

Protection from homologous and heterologous influenza strains with different vaccine platforms highlights that M2e-induced humoral responses trigger the broad range of protection against lethal outcomes reported in chicken, mouse, ferret, rhesus monkey, and rabbit models. Additionally, passive transfer studies performed in mice using purified monoclonal antibodies or sera from vaccinated animals had led to a significant survival rate of passively immunized mice after challenge with lethal IAV (Fan et al., 2004; Jegerlehner et al., 2004; Schotsaert et al., 2009). Whether protection was modulated by passive immunity or antibody induction by active immunization, sera were not capable of neutralizing virus, suggesting M2e specific antibodies function through other mechanisms to convey animal protection. The mechanism of protection conferred by M2e vaccines will be discussed in a later section.

2. **M2e-based vaccines in chicken**

There has been extensive work with M2e vaccines in mice. However, limited research had been done on natural host of IAV such as chicken and pigs. As our focus in this thesis is developing vaccines against AI in chickens, we will review the different M2e constructs used for vaccination in chickens. In one of the first studies performed in
chickens, birds were vaccinated twice with different constructs of *Salmonella Enteritidis* expressing M2e epitope in combination with a potential immune-enhancing CD154 peptide sequence. The results showed that vaccinated chickens exhibited significantly increased M2e-specific IgG responses plus simultaneous expression of CD154 enhanced the immune response. Upon challenge of vaccinated birds with either LPAI or HPAI viruses, the birds were protected against LPAI viruses but not against HPAI virus. This data indicated that M2e-based vaccines was immunogenic in chickens and protected immunized birds against at least LPAI (Layton et al., 2009). Zhang et al., (2011) highlighted the importance of multiple M2e copies presentation on the carrier in order to improve the immunogenicity of M2e vaccines in chickens (Zhang et al., 2011). Presentation of multiple M2e copies enhanced the immune response in vaccinated birds but not in a linear fashion as 3 copies construct showed both higher antibody titers and better protection against HPAI challenge compared to other constructs used.

Two nanoparticle vaccine constructs expressing one or four copies of M2e were used to immunize chickens. Four copies of M2e enhanced anti-M2e specific IgG titers compared to one copy construct. Moreover, both constructs induced significantly higher M2e-specific response compared to a non-vaccinated group. M2e vaccinated sera were not able to show neutralization activity against H5N2 by plaque reduction assay indicating that mechanism of protection in chicken might not be the same as mice. When the birds were challenged with LPAI virus, the four copy construct were able to reduce
the challenge virus shedding titers form both tracheal and cloacal swabs. The immune response and protection observed might indicate improvement of the immune response against M2e in chicken but still there was no protection observed against HPAI virus (Babapoor et al., 2011).

A live *Lactococcus lactis* expressing 10 tandem copies of avian M2e protein (LL-M2e) or keyhole-limpet-hemocyanin conjugated M2e (KLH-M2e) was used to vaccinate chickens. Intranasal vaccination with LL-M2e or subcutaneous vaccination with KLH-M2e induced high M2e-specific immune response compared to non-vaccinated birds. The high immune response correlated with prolonged survival rate and reduced viral shedding from tracheal samples after challenge with HPAI. However, there was undetectable M2e specific CD4+ T cell response or fecal IgA in LL-M2e vaccinated birds. Overall, survival and protection observed after vaccination were due to serum M2e-specific IgG response (Reese et al., 2013).

One of the few M2e vaccine studies in chicken that evaluated the cell mediated immune response after vaccination with a recombinant M2e protein fused to HSP70 (M2e/HSP70) (Dabaghian et al., 2014). The birds after twice immunization with fusion protein construct induced significantly high anti-M2e antibodies compared to M2e alone group. Additionally, an enhanced T-cell proliferative response, increased production of IFN-γ (Th1-type) and IL-4 (Th2-type) cytokines, and significantly increased the percentages of CD4+ and CD8+ T-cells were detected M2e/HSP70 vaccinated birds.
compared to non-vaccinated group. While chickens vaccinated with IIV or adjuvant alone did not show any response after stimulation with synthetic M2e peptide. With regards to protection against LPAI H9N2 challenge, the birds immunized with M2e/HSP70 had significantly lower oropharyngeal and cloacal viral shedding than did the challenged control, M2e alone or IIV vaccinated birds. Overall, this study highlighted the importance of the carrier (HSP70) to enhance the immunogenicity of M2e plus induction of both cellular and humoral immunity to confer improved protection against IAV challenge (Dabaghian et al., 2014).

**Mechanisms of M2e vaccine conferred protection against influenza**

Understanding the mechanism of protection by M2e-based vaccines is critical for design of vaccine constructs and development of new platforms. Previous studies based on M2e vaccine have monitored the development of M2e specific antibodies and demonstrated that IgG antibodies are essential for protection against IAV challenge. This is consistent with studies showing that animals, especially mice, can be protected against challenge by injection of either M2e-specific monoclonal antibodies (Ernst et al., 2006; Jegerlehner et al., 2004; Tompkins et al., 2007) or M2e-vaccine derived immune serum (Fan et al., 2004; Neirynck et al., 1999; Wu et al., 2007a).

It was proposed that M2e-specific antibodies would disturb the interactions between both M1 and M2 proteins and subsequent interaction of the M1 protein with the HA, NA proteins, and the nucleocapsid complexes, thus perturbing virus assembly and
causing growth restriction (Zebedee and Lamb, 1989). Moreover, anti-M2e antibodies could efficiently recognize and bind their native antigens expressed on virus-infected cells, proposing indirect mechanism of protection. This mechanism require the cooperation of four different immunological factors: NK cells, Fc receptors (FcR), complement, and T cells. Thus, preventing the release of viral particles into the extracellular fluids, and/or enhancing the uptake by phagocytic cells through NK cell ADCC and complement-dependent cytolysis.

Earlier studies with M2e vaccines had proposed the role of NK cells in conferring protection against IAV challenge through ADCC. Depletion of NK cells before lethal challenge had abrogated the protection in immunized mice (Jegerlehner et al., 2004). On the contrary, another studies showed that M2e mediated protection was independent of NK cell role and involved different immune mechanisms (De Filette et al., 2006b; Fu et al., 2009a). Thus, it is likely that NK cells may contribute to M2e immune mediated protection via multiple mechanisms.

Previous reports have highlighted the critical role of FcR mediated phagocytosis by macrophages in clearance of IAV infected cells (Hashimoto et al., 2007; Huber et al., 2001). Previous studies have shown that anti-M2e antibodies passively transferred could mediate protection against influenza infection in vivo (Neirynck et al., 1999; Zharikova et al., 2005). Additionally, FcR common γ chain expressed on alveolar macrophages was identified as crucial role in protection as shown by elimination of IAV-infected cells (El
Bakkouri et al., 2011). Taken together, anti-M2e antibodies enhance the uptake of antibody-bound virions via DCs and macrophages forming immune complexes that would further contribute to increasing antiviral IFN-γ-secreting T cell responses.

In mice, M2e specific IgG isotypes induced by vaccination are to a certain extent predictive for the protective efficacy (Pejoski et al., 2010; Vasin et al., 2014). For instance, IgG2a responses reflect a Th1 type of immune response which correlate with better protection compared to IgG1. In a previous study, mice vaccinated with M2e-HBc induced significant levels of IgG2a/IgG1 responses and improved protection against viral challenge were obtained (Ramos et al., 2014). This improved protection is attributed to the ability of IgG2a antibodies for the activating receptors FcγRI, III and IV, whereas mouse IgG1 only binds significantly to FcγRIII (Song et al., 2014). In chickens, no previous studies were performed to demonstrate the role of FcR in M2e mediated protection. In birds, the predominant circulating antibody is IgY is recognized by the high-affinity FcY receptor (chicken Ig like receptor (CHIR)-AB1). CHIR-AB1 binds IgY in a similar way as human FcαRI binds IgA. The IgY receptor is expressed on chicken B cells, macrophages, monocytes and NK cells (Pürzel et al., 2009; Viertlboeck et al., 2007). Extrapolating the findings on the protective mechanism of M2e-based immunity from mice to birds would mean that only full length IgY contributes to protection.

Complement can play a vital role in virus elimination as previous reports have shown that it can bind to IAV in the presence of virus specific antibodies. The role of
complement in M2e-antibodies mediated protection has been controversial as well as the role of NK cells. Passive transfer of human anti-M2 monoclonal antibodies in C3 knockout mice was able to significantly reduce the lung viral loads of infected animals (Wang et al., 2008c). However, Jegerlehner et al. (2004) demonstrated that the complement component C3 is not critical for anti-M2e antibody-mediated protection by intraperitoneal passive transfer of M2e immune sera. This discrepancy might be due to the differences in experimental protocols as vaccine type, vaccination procedure and isolation of immune cells. Further studies are needed to better understand the possible roles of complement C3 in conferring protection by using an appropriate vaccine that can elicit a T helper type 1-biased antibody immune response in mice and also in chickens.

T cell immunity plays an important role during recovery form IAV infection especially when M2e vaccines are able to induce a T cell response. M2e specific CD4+ but not CD8+ T cell response was induced in mice after vaccination with M2e fusion protein (Eliasson et al., 2008). Additionally, depletion studies in mice have been used to determine the role of T cell in conferring protection. Vaccination of mice with M2 DNA and treatment with CD4+, CD8+, or CD4+ and CD8+ antibodies prior to viral challenge have shown that depletion of both CD4+ and CD8+ T cell but not either single CD4+ or CD8+T cell showed significant loss of efficacy and survival indicating the roles of CD4+ and CD8+ T cells in conferring M2 immunity (Tompkins et al., 2007). Although it is not clear why there is discrepancy in T cell responses among different studies, differences in
immunization protocols such as the platforms of vaccines and adjuvants, strains of animals, routes of immunization and with or without adjuvants may affect the differential outcomes of immune responses.

**Supplemented vaccination of IIV and M2e**

The development of a cross reactive IAV vaccine had been the desired goal for long time. Despite the high variability of the 2 major surface proteins (HA and NA), studies using different conserved proteins (M2, HA2, M1, NP) revealed their ability to provide heterosubtypic protection against different IAV with their consideration as target antigens for the development of universal vaccine (Du et al., 2010; Pica and Palese, 2013). However, the conserved antigenic targets suffer from their relatively lower immunogenicity and efficacy compared to HA-based vaccines.

M2e-based vaccines require multiple immunizations in the presence of potent adjuvants, carriers to enhance their immunogenicity. Additionally, since M2e antibodies do not directly neutralize IAVs, the protective efficacy by M2e antibodies is lower than that of HA-based IIV that induce neutralizing antibodies against homologous strains. Therefore, it is unlikely that M2e-based vaccines would be developed as a standalone universal vaccine which would completely replace current HA based influenza vaccines. A more realistic approach for inducing broader cross protection would be supplementation of current influenza vaccines with highly conserved antigens such as M2e to improve the limited cross protective efficacy of current vaccines. For that regard,
several studies in either mice or chickens had been performed (Kim et al., 2013a; Kim et al., 2014b; Park et al., 2014; Song et al., 2016; Song et al., 2011a; Wu et al., 2009). In mice, combination of M2e, either as VLP or recombinant protein, with IIV or split vaccine provided cross protection against lethal challenge with heterologous and heterosubtypic viruses in terms of reducing weight loss and lung viral shedding titers and providing long-term cross protective immunity (Kim et al., 2014b; Lee et al., 2015; Song et al., 2011a).

In chickens, supplementation of recombinant M2e protein to inactivated vaccine prepared from H9N2 virus provided an additive effect in reducing heterologous H9N2 challenge viral titers from tracheal and cloacal swabs and viral replication in trachea and cecal tonsils (Park et al., 2014). In another study, chickens vaccinated with inactivated vaccine prepared from H5N1 virus, supplemented with M2e-5x VLPs were protected against lethal challenge with heterologous H5 HPAI viruses, plus induction of M2e specific immune response that cross reacted with different viruses regardless of HA subtype (Song et al., 2016). Thus, combined vaccination (IIV and M2e) offer an advantage of overcoming the limited cross reactivity of IIV with the ability to confer protection against heterosubtypic and heterologous IAV. Thus, supplementation of M2e antigen to IIV along with other control measures can play an important role in controlling novel emerging viruses.
1.5. **Norovirus as a vaccine platform for M2e**

The recombinant vaccine technology has enabled the production of custom made seed stains against AI. They offer rapid, scalable production and safety due to absence of infectious viral material. The whole or portions of viral capsid proteins can be easily expressed *in vitro* and spontaneously assemble into small nanoparticle. Those complex multivalent particles still retain their native antigenic properties and therefore make them possible candidates for subunit vaccines. The norovirus P particle is considered one of those subviral nanoparticles that have shown promising results as a successful platform for different antigens (Tan et al., 2011; Tan and Jiang, 2012).

Norovirus (NoV) is a non-enveloped virus with an outer protein consisting solely of one major structural protein, the capsid protein. The capsid protein consists of two major domains; the shell (S) and the protruding (P) domain, which are linked together by a short flexible hinge. The S domain is involved in icosahedral shell formation, whereas the P domain forms the arch-like dimeric structure that extends from the shell. The P domain can be further divided into two subdomains; P1 and P2. The P1 subdomain forms the leg of the P dimer connecting it to the S domain, while the P2 subdomain is located on the outermost surface and involved in binding of norovirus to human histo blood group antigen (Tan et al., 2003; Tan et al., 2008b; Tan et al., 2009).

*In vitro* expression of the P domain in *E. coli* forms three different P domain complexes: the 24-mer P particle (~20 nm), the 12-mer small P particle (~14 nm), and the
P dimer (~6 nm). The P domain complexes are interchangeable with the P dimer as the building block. 3D structure reconstruction of the P particle by electron cryomicroscopy revealed a nanoparticle with an octahedral symmetry with the P dimer as the building block. The P particle appears spherical having a center cavity and 12 P dimer spikes protruding outward. Additionally, the crystal structure of the norovirus P protein indicated three loops on the distal surface of each P domain corresponding to the outermost surface of the P particle (Fig. 1.1). These loops are potential sites for foreign antigen presentation. Since each P monomer has three surface loops, insertion of a foreign antigen into these loops results in 24 to 72 copies of the antigen on the surface of a P particle, which could greatly enhance the antigenicity and immunogenicity of the antigens. Thus, the P particle is an excellent Nano carrier for foreign antigen presentation (Tan et al., 2011).

Among the 3 surface loops, loop 2 has been extensively studied and several antigens of different sizes have been inserted, like VP8 protein of rotavirus and M2e of IAV. These studies led to development of two P particle-based bivalent vaccine candidates against NoV and rotavirus, as well as NoV and IAV. Both vaccines have been examined in animal models and demonstrated strong immune response and protection against infection of corresponding pathogens, providing a new concept of a dual vaccine against two infectious diseases.
The M2e epitope was also inserted into loop 2 of the P particle and the resulting P particle–M2e chimera (M2e-PP) induced significantly higher M2e-specific antibodies in comparison to that induced by the free M2e peptide. Moreover, M2e-PP provided 100% protection in mice against lethal challenge of a mouse-adapted IAV (PR8 strain; H1N1), which was significantly higher than that provided by the free M2e epitope. Regarding the antibody isotypes to the chimeric vaccine, IgG1 titer was induced to high titers similar to that of the total IgG, suggesting that the chimeric vaccine induced a strong Th2-type response and that IgG1 may play an important role in the protective immunity. As expected, the chimeric vaccine also induced high antibody response to the P particle platform and the antibody strongly inhibited binding of NoV VLP to viral receptors. These data showed that the M2e-PP chimera is a promising dual vaccine against both the NoV and IAV (Xia et al., 2011).

The P particle is stable, highly immunogenic, and able to induce a neutralizing antibody against NoV. It also offers another advantage as a vaccine platform through insertion of foreign antigens into one of the three surface loops of each P domain resulting in new chimeric P particles with enhanced immunogenicity of the inserted antigens.
Figure 1.1. Norovirus P particle expression and presentation of foreign antigens in their context. (A) The dimeric norovirus capsid protein VP1 consists of an S domain and a P domain, with the P domain divided into two subdomains; P1 and P2. In vitro expression of P domain leads to the formation of P dimer that can further assemble into the 12-mer small P particle and 24-mer P particle. (B) The structure of norovirus P particle of VA387 (GII.4) reconstructed by cryo-EM (left), The distal end of a protrusion of the P particle with the 3 surface loops are illustrated (center), and an example of epitope after insertion in loop 2 (right). Pictures are adapted from (Bereszczak et al., 2012; Tan and Jiang, 2012).
<table>
<thead>
<tr>
<th>Vaccine Platform</th>
<th>Model</th>
<th>Humoral response</th>
<th>T cell involved</th>
<th>Mechanism of protection</th>
<th>Reference</th>
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<tbody>
<tr>
<td>M2e-HBc</td>
<td>Mice</td>
<td>IgG1, IgG2a, IgA</td>
<td>CD4⁺</td>
<td>NK ADCC</td>
<td>(El Bakkouri et al., 2011; Jegerlehner et al., 2004; Neirynck et al., 1999)</td>
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<tr>
<td>STF2.4xM2e</td>
<td>Mice</td>
<td>IgG1, IgG3</td>
<td>ND</td>
<td>ND</td>
<td>(Huleatt et al., 2008; Talbot et al., 2010)</td>
</tr>
<tr>
<td>DNA plasmid with M1 and M2</td>
<td>Mice</td>
<td>IgG, IgA</td>
<td>CD8⁺ &amp; CD4⁺</td>
<td>ND</td>
<td>(Okuda et al., 2001)</td>
</tr>
<tr>
<td>Synthetic M2e</td>
<td>Mice</td>
<td>IgG1, IgM, IgG2b, IgG2a, IgG3, IgA</td>
<td>CD4⁺, IL-4, IFNγ</td>
<td>Cell to cell inhibition</td>
<td>(Wu et al., 2007a)</td>
</tr>
<tr>
<td>Baculovirus-M2</td>
<td>Mice</td>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
<td>(Slepushkin et al., 1995)</td>
</tr>
<tr>
<td>M2e-bovine serum albumin</td>
<td>Rabbit</td>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
<td>(Liu et al., 2003; Liu et al., 2004b)</td>
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Table 1.1. Continued

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<tr>
<th>Vaccine Platform</th>
<th>Model</th>
<th>Humoral response</th>
<th>T cell involved</th>
<th>Mechanism of protection</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>M2e linked to Neisseria meningitides outer membrane complex or keyhole limpet hemocyanin</td>
<td>Monkey, Ferret, Mice</td>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
<td>(Schotsaert et al., 2009)</td>
</tr>
<tr>
<td>CTA1-M2e-DD</td>
<td>Mice</td>
<td>IgG1, IgG2a, IgA</td>
<td>CD4⁺ &amp; IFNγ⁺</td>
<td>ND</td>
<td>(Eliasson et al., 2008)</td>
</tr>
<tr>
<td>M2 DNA/ Adenovirus</td>
<td>Mice</td>
<td>IgG</td>
<td>CD4⁺ &amp; CD8⁺⁺</td>
<td>ND</td>
<td>(Tompkins et al., 2007)</td>
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<tr>
<td>M2e-5x VLP</td>
<td>Mice</td>
<td>IgG1, IgG2a</td>
<td>IFN-γ⁺</td>
<td>ND</td>
<td>(Kim et al., 2013b)</td>
</tr>
<tr>
<td>M2e-5x VLP</td>
<td>Mice</td>
<td>IgG</td>
<td>CD4⁺ &amp; CD8⁺⁺</td>
<td>ND</td>
<td>(Lee et al., 2014b)</td>
</tr>
<tr>
<td>Recombinant Tobacco mosaic virus</td>
<td>Mice</td>
<td>IgG1, IgG2a</td>
<td>ND</td>
<td>ND</td>
<td>(Petukhova et al., 2013)</td>
</tr>
<tr>
<td>Vaccine Platform</td>
<td>Model</td>
<td>Humoral response</td>
<td>T cell involved</td>
<td>Mechanism of protection</td>
<td>Reference</td>
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<td>Malva mosaic virus nanoparticles</td>
<td>Mice, Dogs</td>
<td>IgG, IgG2a</td>
<td>ND</td>
<td>ND</td>
<td>(Leclerc et al., 2013)</td>
</tr>
<tr>
<td>Papaya mosaic virus VLP</td>
<td>Mice</td>
<td>IgG1, IgG2a, IgG2b, IgG3</td>
<td>ND</td>
<td>ND</td>
<td>(Denis et al., 2008)</td>
</tr>
<tr>
<td>Synthetic M2e-multiple antigen peptide</td>
<td>Mice</td>
<td>IgG, IgA</td>
<td>ND</td>
<td>ND</td>
<td>(Mozdzanowska et al., 2003; Wolf et al., 2011)</td>
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<tr>
<td>M2e linked rotavirus NSP4</td>
<td>Mice</td>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
<td>(Andersson et al., 2012)</td>
</tr>
<tr>
<td>M2e-tGCN4</td>
<td>Mice</td>
<td>IgG1, IgG2a</td>
<td>ND</td>
<td>ND</td>
<td>(De Filette et al., 2008a)</td>
</tr>
<tr>
<td>M2e-PP</td>
<td>Mice</td>
<td>IgG, IgG1, IgG2b</td>
<td>ND</td>
<td>ND</td>
<td>(Xia et al., 2011)</td>
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<tr>
<th>Vaccine Platform</th>
<th>Model</th>
<th>Humoral response</th>
<th>T cell involved</th>
<th>Mechanism of protection</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>4×M2e HSP70 fusion protein</td>
<td>Chicken</td>
<td>IgG</td>
<td>IFN-γ, IL-4, CD4⁺: CD8⁺</td>
<td>ND</td>
<td>(Dabaghian et al., 2014)</td>
</tr>
<tr>
<td>Recombinant M2e protein</td>
<td>Chicken</td>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
<td>(Swinkels et al., 2013)</td>
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<tr>
<td>Recombinant M2e protein Multiple</td>
<td>Chicken</td>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
<td>(Zhang et al., 2011)</td>
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<tr>
<td>copies</td>
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<tr>
<td>M2e expressed on nanoparticle</td>
<td>Chicken</td>
<td>IgG</td>
<td>ND</td>
<td>ND</td>
<td>(Babapoor et al., 2011)</td>
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</tbody>
</table>

ND: not determined
1.6. References


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Chapter 2: Immunogenicity and Protective Efficacy of the Norovirus P particle-M2e Chimeric Vaccine in Chickens

2.1. Abstract

The ectodomain of the influenza matrix protein 2 (M2e) is highly conserved across strains and has been shown to be a promising candidate for universal influenza vaccine in the mouse model. In this study, we tested the immunogenicity and protective efficacy of a chimeric norovirus P particle containing the avian M2e protein against challenges with three avian influenza (AI) viruses (H5N2, H6N2, H7N2) in chickens. Two-week-old specific pathogen free chickens were vaccinated 3 times with an M2e-P particle (M2e-PP) vaccine via the subcutaneous (SQ) route with oil adjuvant, and transmucosal routes (intranasal, IN; eye drop, ED; microsprayer, MS) without adjuvant. M2e-PP vaccination via the SQ route induced significant serum IgG antibody responses which were increased by each booster vaccination. In groups vaccinated via IN, ED or MS, neither IgG nor IgA antibody response was detected from sera or nasal washes of immunized birds. The M2e-PP vaccination via the SQ route significantly reduced the virus shedding in the trachea and the cloaca for all three challenge viruses. Despite the absence of detectable IgG and IgA responses in birds vaccinated with the M2e-PP via intranasal route, a similar level of reduction in virus shedding was observed in the IN
group compared to the SQ group. Our results support that the universal vaccine approach using M2e based vaccine can provide cross-protection against challenge viruses among different HA subtypes although the efficacy of the vaccine should be enhanced further to be practical. A better understanding of the protective immune mechanism will be critical for the development of an M2e-based vaccine in chickens.

2.2. Introduction

Influenza A viruses belong to the *Orthomyxoviridae* family which possesses a negative-sense, single-stranded, and segmented RNA genome. Influenza A viruses can be further subtyped according to the antigenic properties of hemagglutinin (HA) and neuraminidase (NA) subtypes (Palese and Shaw, 2007). There are 16 HA and 9 NA subtypes detected in poultry and wild birds worldwide (Fouchier et al., 2005; Webster et al., 1992). Recently, H17N10 and H18N11 viruses were detected from bats (Wu et al., 2014). Influenza A viruses infect a wide range of hosts including humans, poultry and swine leading to seasonal epidemics or pandemics (Molinari et al., 2007). In poultry, avian influenza (AI) virus causes a wide range of symptoms from asymptomatic infection with the low pathogenic AI (LPAI) virus to severe systemic infection with 100% mortality with the highly pathogenic AI (HPAI) strains (Swayne and Halvorson, 2008). The economic losses associated with AI in poultry are either directly due to mortality or indirectly due to costs of prevention and control of disease (Swayne, 2008). Increasing the resistance of chickens against AI virus will not only prevent the economic losses for
the poultry industry but will also decrease the public health risk by reducing both viral shedding and transmission (Swayne and Kapczynski, 2008b).

Vaccination with inactivated whole virus vaccines remains the major preventive measure for AI. Currently, all commercial vaccines are targeting the surface proteins, especially HA, to induce protective neutralizing antibodies (Kapczynski and Swayne, 2009). However, the HA protein is highly variable across the strains and inactivated vaccines in general provide good protection against homologous (antigenically similar to vaccine strain) challenge but not against the heterologous (antigenically different strains within same subtype) or heterosubtypic (different HA subtypes) challenges (Johansson and Brett, 2007; Kapczynski and Swayne, 2009; Lee and Suarez, 2005; Swayne and Kapczynski, 2008a; Swayne and Kapczynski, 2008b, 2009; Zhang et al., 2012). For this reason, new approaches and strategies to develop broadly reactive vaccines are urgently needed.

Recent studies showed that conserved proteins of influenza virus can provide protective immune responses against heterologous or heterosubtypic influenza viruses (Du et al., 2010). These conserved domains include the matrix 2 (M2), hemagglutinin 2 (HA2) and some other structural proteins. The extracellular domain of the M2 protein (M2e) consists of 23 amino acids which are remarkably conserved among influenza subtypes. Comparison of M2e sequence from different human, swine and avian viruses revealed that N-terminal amino acids are identical among these viruses and only 5 amino
acids at position 10-20 were found to be host specific (Ebrahimi and Tebianian, 2011). Because M2e is small in size and poorly immunogenic as it is, several approaches were developed to increase the immunogenicity including; fusion of the M2e peptide to the Hepatitis B virus core protein (De Filette et al., 2008b), Heat Shock Protein-70 of M. tuberculosis (Dabaghian et al., 2014; Ebrahimi et al., 2010), and expressing through influenza virus-like particles (Song et al., 2011b). These constructs have induced an immune response against M2e which in turn showed varying degrees of protection in different animal models against different challenge strains. Some earlier studies in mice showed a close relationship between antigen dose or epitope density in one single molecule and the humoral response which in turn could estimate its protective efficacy (Liu et al., 2004a). They showed that high M2e epitope density fused to glutathione transferase enhanced the M2e antibody response which protected mice against lethal challenge. Most studies conducted using mouse model supported the M2e as a promising target for development of universal influenza vaccines.

In poultry, different M2e-based vaccines have been tested for the last 5 years to evaluate their efficacy against LPAI or HPAI virus challenge. In an earlier study, a Salmonella enteritidis strain expressing a conserved region of M2e (amino acid 6-13) in association with a CD154 ligand (a member of tumor necrosis factor ligand family expressed on activated T-cells) was used for chicken immunization using a prime boost regime. Vaccinated chickens showed protection against LPAI H7N2 challenge in terms
of reduction in viral shedding but did not reduce the clinical signs, morbidity and mortality to HPAI H5N1 (Layton et al., 2009). The *Lactococcus lactis* expressing M2e on the surface also showed minimal protective efficacy against H5N2 HPAI virus challenge (Reese et al., 2013). Infectious bursal disease virus (IBDV) was used to incorporate M2e into the VP2 of the virus, and the chimeric vaccine showed poor efficacy in inhibiting H9 virus replication post challenge (Tang et al., 2013). A novel platform utilizing a self-assembling polypeptide nanoparticle was used to present M2e in either monomeric or tetrameric forms. The constructed vaccine, especially in tetrameric form, showed significant reduction in virus shedding against H5N2 LPAI virus challenge (Babapoor et al., 2011). Most recently, vaccination of chickens with four copies of M2e linked to *Mycobacterium tuberculosis* HSP70 were shown to enhance both humoral and cell-mediated immune responses and reduce viral shedding against H9N2 LPAI virus challenge (Dabaghian et al., 2014).

In this study, we utilized a norovirus P particle platform expressing 24 copies of the M2e that has been found to be highly immunogenic and provide good protection against lethal influenza virus challenge in the mouse model (Xia et al., 2011). An AI virus consensus M2e sequence expressed on the surface loop of the P particle was constructed and the immunogenicity and protective efficacy against 3 different subtypes of LPAI viruses were evaluated in chickens.
2.3. Material and methods

2.3.1. Viruses

The challenge virus strains used in this study, A/Chicken/PA/13609/93 (H5N2), A/Chicken/CA/431/00 (H6N2), and A/Chicken/NJ/150383-7/02 (H7N2), were obtained from the repository of the Food Animal Health Research Program (Wooster, OH) and were passaged once in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECEs) to prepare working stocks for the study.

2.3.2. Construction and confirmation of M2e-P particle (M2e-PP) chimeric vaccine

The M2e-PP chimeric protein containing the AI virus consensus M2e peptide sequence (MSLLTEVETPTRNGWECKCSDSSD) was constructed using a P-particle expression vector [pGEX-4T-1 containing P-domain sequence of norovirus VA387, genogroup II, cluster 4(GII.4)] as described previously (Xia et al., 2011). Molecular weight of the vector (VA387 P particle) is 36.06 KDa and the M2e is 4.18 KDa which results in ratio of 8.63:1 for vector and M2e. To confirm the P particle formation, gel filtration chromatography was carried out through an AKTA FPLC System (GE Healthcare Life Sciences, Pittsburgh, PA) as described previously (Xia et al., 2011). Briefly, the affinity column-purified proteins were loaded on a size exclusion column Superdex 200 (GE Healthcare Life Sciences, Pittsburgh, PA) powered by an AKTA
FPLC system. The molecular weights of the eluted fractions were calibrated by Gel Filtration Calibration Kits (GE Healthcare Life Sciences, Pittsburgh, PA).

2.3.3. Vaccination and challenge of birds

In the first experiment, two-week-old SPF chickens were divided into 2 major groups according to the vaccination route: subcutaneous (SQ) route with adjuvant (Montanide ISA 70, Seppic, France) mixed in 3:7 volume ratio and intranasal (IN) routes without adjuvant. Birds in each major group were divided into 5 subgroups and received 4 different doses (5, 10, 50, 200 µg/bird) of M2e-PP (n=4 for 5, 10, and 50 dose groups; n=2 for 200 µg dose group) or P particle without M2e in a dose of 10 µg/bird (n=2 for SQ route; n=3 for IN route group). Another group of 2 birds served as the negative control. Chickens were vaccinated three times with 2 week intervals between each vaccination. Sera were collected 2 weeks after each immunization to evaluate the M2e specific IgG titers by ELISA.

In the second experiment, two-week-old SPF chickens were vaccinated with M2e-PP (5µg/bird) via the SQ route with adjuvant. Birds were also vaccinated via 3 different transmucosal routes without the addition of adjuvant: direct intranasal drop (IN), eye drop (ED), and using microsprayer (MS). The dose was determined based on the result from the first experiment which showed higher serum IgG titers in birds vaccinated with 5 µg of the M2e-PP compared to the use of 50 µg /bird or 200 µg /bird. To further determine the efficacy of lower doses of M2e-PP, two additional groups of birds were
vaccinated 3 times with 1 and 3 µg/bird of M2e-PP via the SQ route with adjuvant (Table 2.1). Another two groups of birds received 5 µg of P-particle control and PBS, respectively. Sera were collected 2 weeks after each immunization to evaluate the IgG responses. Nasal washes were collected from 3 birds from the IN, ED, MS and PBS immunization groups to evaluate the IgA responses. Two weeks after the third immunization, birds were challenged through the intranasal route with 10^6 EID_{50}/0.2 ml of H5N2 and H7N2, respectively. Another three groups of birds vaccinated via the SQ route with M2e-PP, P particle control and PBS, respectively, were challenged with 10^6 EID_{50}/0.2 ml of H6N2 AI subtype (Table 2.1).

Tracheal and cloacal swabs were collected from the infected birds at 2 and 4 days post inoculation (DPI). At 10 DPI, all birds were bledd to determine the hemagglutination inhibition (HI) titers (Lee et al., 2004). Swab samples were processed as previously described (Ali et al., 2013). Briefly, swab samples were vortexed and then centrifuged at 2000 rpm for 10 min at 4°C to pellet the debris. The RNA was extracted from 100 µl of the swab sample supernatant using a Viral RNA Extraction Kit® (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Viral genome amounts were determined by quantitative real-time RT-PCR using influenza A virus matrix gene specific oligonucleotide primers and probes and converted to viral titers as previously described (Lee and Suarez, 2004).
2.3.4. Enzyme-linked immunosorbent assay (ELISA) for serum antibody titer determination

ELISA was performed as previously described (Xia et al., 2011) with modifications. Briefly, synthesized free M2e peptides (MSLLTEVETPTRNGWECRCSDSSD, 2 µg /ml, Ohio peptide, Powel, OH) and purified P particle were used as ELISA antigens for M2e or P particle specific antibodies, respectively. Sera and nasal washes were initially diluted to 1:50 and then 2 fold serial dilutions were made in 2.5% non-fat dry milk in PBS (pH 7.4) to determine the end-point titers. Ninety-six well microtiter plates (Nunc MaxiSorp®, USA) were coated with antigens (200 ng/well) at 4°C overnight. Next day, plates were incubated at room temperature for 2 h. The wells were then blocked with 200 µl of 5% nonfat dry milk in PBS and then incubated for 2 h at room temperature. After washing, sera (100 µl) were added to the coated plates for 2 h at room temperature. Plates were then washed six times with PBS (pH 7.4) and either goat anti-chicken horse reddish peroxidase (HRP) labeled IgG (KPL, Gaithersburg, MD) or IgA conjugates (Novus Biologicals, LLC, Littleton, CO) were added. After incubating the plates for 2 h at room temperature, the plates were washed six times followed by addition of the substrate, tetramethylbenzidine peroxidase (TMB; KPL, Gaithersburg, MD) (50 µl /well), and the plates were incubated for 15 min at room temperature. The reaction was stopped by adding 50 µl/well of 1M phosphoric acid, and plates were read at 450 nm (i-Mark ELISA Reader, Bio-rad, Life Science)
Research, CA, USA). Sera and nasal washes from PBS vaccinated group were used as controls. Antigen-specific antibody titers were defined as the reciprocal of the highest dilution with an OD 450 that is above the mean of the negative control sera plus two standard deviations.

2.3.5. Statistical analysis.

Statistical analyses were performed using one way analysis of variance (ANOVA) followed by Tukey’s post-hoc test to estimate differences among virus titers and HI antibody titers using Graph Pad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

2.4. Results

2.4.1. P particle formation of the P domain-M2e chimera characterized by a gel filtration chromatography

A gel filtration chromatography of the chimeric P domain-M2e protein was performed (Fig. 2.1A) and the fractions were analyzed by SDS-PAGE (Fig. 2.1B). The results showed that the vast majority (>95%) of the chimeric proteins formed a defined peak at ~910 KDa, indicating that the chimeric protein formed P particles like those obtained in our previous studies in presentation of variable small peptide antigens.
2.4.2. The M2e-specific antibody responses in chickens vaccinated with M2e-PP chimera

The anti-M2e immune response was monitored by determining the M2e-specific IgG titer in the sera collected at 2 weeks after each immunization. Chickens vaccinated with M2e-PP via the SQ route with adjuvant developed detectable serum IgG levels after the first vaccination which was increased after each booster vaccination (Fig. 2.2A). The ELISA titer based on end-point dilution of the sera showed that the lower doses used in this experiment (i.e., 5 and 10 µg/bird) induced a higher level of M2e antibody when compared to the two higher doses (50 and 200 µg groups) (Fig. 2.2A, top). In contrast, the antibody response against the P particle was dose dependent and higher titers were observed in 50 and 200 µg dose groups compared to the 5 µg dose group (Fig. 2.2A, bottom). For the IN groups, we were not able to detect IgG or IgA in any of the vaccinated birds except for a minimal IgG titer observed in birds vaccinated with the 200 µg dose after the third vaccination.

In the second experiment, based on the results obtained from the first experiment, two additional doses (1 and 3 µg /bird) of M2e-PP were tested in comparison to the 5 µg/bird dose. The M2e antibody response after each vaccination showed an increase in IgG titer by M2e-PP boosting as observed in the first experiment. In addition, both of the two lower dose groups (1 and 3 µg /bird) showed comparable serum IgG titers to that of the 5 µg /bird dose (Fig. 2.2B).
In birds vaccinated via the three transmucosal routes (IN, ED, and MS), neither IgG in the sera nor IgA in the nasal washes was detected at all time points tested. In our previous study, intranasal vaccination of M2e-PP containing human influenza virus consensus M2e sequence in mice induced a significant IgG titer in the sera (Xia et al., 2011).

2.4.3. **Protective efficacy of M2e-PP vaccine against 3 different subtypes of LPAI virus challenges.**

The protective efficacy and the spectrum of the specific M2e antibody responses induced by M2e-PP chimera were evaluated by the level of virus shedding reduction after challenge with LPAI strains. Two-weeks after the third vaccination, birds were challenged with low pathogenic H5, H6 or H7 subtype strains. Virus titer in tracheal and cloacal samples collected at 2 and 4 DPI were determined using real-time RT-PCR.

Overall, chickens vaccinated with the 5 µg dose of M2e-PP via the SQ route with adjuvant showed a significant reduction in virus shedding after challenge with H5N2, H7N2, and H6N2 viruses (P<0.05) (Fig. 2.3). In birds challenged with H5N2 and H7N2 viruses, we observed a decrease in viral shedding from both tracheal and cloacal swabs in birds vaccinated via the IN route as well as the SQ route. The significant decrease in viral titers was observed in both the SQ and IN groups at both time points (i.e., 2 and 4 dpi) following H5N2 challenge (Fig. 2.3A). Against H7N2 challenge, a significant reduction in viral titers was observed in the cloacal swabs of SQ vaccinated groups at 2 and 4 DPI.
and in tracheal swabs at 4 DPI in both SQ and IN vaccinated groups, respectively (P<0.05) (Fig. 2.3B). Against H6N2 challenge, a significant reduction in viral titers was observed at 2 dpi in tracheal swabs and at 4 dpi both in tracheal and cloacal swabs (Fig. 2.3C). Intranasal vaccine group was not included in H6N2 challenge study.

The low doses (i.e., 3 and 1 µg /bird) of vaccine also provided similar protection against H5N2 and H7N2 virus challenges as observed with the 5 µg dose although a statistical significance was observed less frequently (Fig. 2.4A and B).

All of the birds seroconverted after challenge. No significant differences were observed in the HI titers measured at 10 DPI; however, HI titers were slightly lower in vaccinated birds compared to challenge control birds which indicates a reduced level of virus replication (Fig. 2.3D and 2.4C).

2.5. Discussion

The ectodomain of the influenza M2 protein (M2e) is one of the promising targets to develop a broad-spectrum influenza vaccine considering its conserved amino-acid sequence across the strains and the capability to induce M2-specific immunity that can prevent viral replication (Neirynck et al., 1999). Though the M2e peptide when presented alone is weakly immunogenic, when it is presented through proper carriers the immunogenicity can be enhanced (Layton et al., 2009). The norovirus P particle-M2e chimera containing 24 copies of human influenza M2e on its surface was found to be immunogenic and protective in mice against a lethal human influenza virus challenge.
The P particle-based platform was also used for the presentation of other antigens (Tan et al., 2011; Tan and Jiang, 2012). In the present study, the same norovirus P particle vaccine platform was utilized for the presentation of the avian influenza M2e epitope and the resulting avian M2e-PP vaccine was tested for both immunogenicity and protective efficacy in chickens. The M2e-PP vaccine induced significant IgG titers when applied via the SQ route with adjuvant which was also increased by booster vaccination even when using doses as low as 1µg/bird. It is interesting to note that SQ vaccination with higher doses (50 and 200 µg) induced a lesser amount of IgG compared to vaccination with lower doses (Fig. 2.1A). The mechanism behind this observation is unclear but it may have to do with high immunogenicity of the vector (P particle) itself which may have overwhelmed the host immune cells. However, the antibody response against the P particle was dose dependent and higher IgG titers were observed in 50 and 200 µg dose groups compared to 5 µg dose group.

No detectable humoral immune responses were observed in intranasal vaccination groups which was different from those observed in a mouse study (Xia et al., 2011). These may be attributed to the differences between the avian and mammalian immune system structure and distribution of cells, where in chickens the primary organ for B-cell differentiation and production is the bursa of Fabricius and the major production site for IgA antibodies is the Harderian gland (Swayne and Kapczynski, 2008a). Though we tried the vaccination via eye drop and microsprayer to better stimulate the Harderian gland, we
were not able to detect any immune response. These results may also emphasize the need for proper adjuvant for intranasal delivery of M2e-based subunit vaccines in poultry.

In terms of protective efficacy, vaccination with the 5 µg/bird dose of M2e-PP via the SQ and direct IN routes significantly reduced virus shedding titers in both tracheal and cloacal swabs from birds challenged with different AI virus subtypes compared to unvaccinated and challenged group. However, the observed protective efficacy of M2e-PP vaccination via SQ or IN routes is lower than oil-adjuvanted whole virus inactivated vaccine in terms of the level of reduction in viral shedding (Swayne et al., 1999). We do not expect that M2e-PP will provide good protection against HPAI viruses in reducing clinical signs or mortality. Previous study also showed poor protection against HPAI H5N1 (Layton et al., 2009). In general, high titer of neutralizing antibody is required to prevent systemic spread of highly pathogenic virus and M2e-specific antibody is considered non-neutralizing. The exact mechanism of the protection by M2e based vaccines remains to be fully elucidated.

The protective efficacy of the M2e-based vaccine in chickens was in general lower than the efficacy that has been shown in the mouse model (De Filette et al., 2006a; Jegerlehner et al., 2004; Schotsaert et al., 2013; Xia et al., 2011). Furthermore, in the mouse model, IN vaccination induced a high IgG response in the sera and provided good protection that was similar to the SQ vaccinated mice (De Filette et al., 2006a; Xia et al., 2011). These different results may be explained by anatomic, physiologic, and
immunologic difference between the avian and mammalian species. Further studies are needed to identify the immune correlates of protection in each species.

In this study, we evaluated the norovirus P particle vaccine platform expressing the avian M2e in the chicken model. The M2e-PP chimeras were immunogenic when administered via the SQ route with adjuvant and could provide protective antibody responses in chickens against different AI virus subtypes. It will be worthwhile to evaluate other possible innate and/or adaptive protection mechanisms induced by M2e in the chicken model. This study shows that the M2e-PP vaccine has the potential to be applied in poultry as one of the components of a universal vaccine in chickens with further improvement in M2e-PP vaccine construct and optimizing the dose and vaccination strategy.

2.6. Acknowledgements

The authors would like to thank Megan Strother for technical assistance. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2013-67015-20476 from the USDA National Institute of Food and Agriculture. Mohamed Elaish is supported by a Ph.D. scholarship from the Egyptian Ministry of Higher Education.
Figure 2.1. Particle formation of the P domain-M2e chimera characterized by gel filtration chromatography. (A) The elution curve of a gel filtration chromatograph of the thrombin-released P-domain-M2 protein using the size exclusion column Sperdex 200. Peaks representing void, P particle-M2e, P dimer-M2e and peak-3 were indicated, respectively. The sizes of the peaks were calibrated with blue dextran 2000 (~2000 kDa, void), wild type P particle (~830 kDa), and wild type P dimer (~70 kDa), respectively. (B) The fractions of the gel filtration chromatography were analyzed by SDS PAGE, the fractions representing three peaks are indicated.
A. Gel filtration chromatography of thrombin released Avian-M2e-PP protein

B. SDS-PAGE analysis of the peaks from gel filtration
Figure 2.2. Serum antibody responses of SPF chickens to the M2e-P particle (M2e-PP) chimera after each vaccination. (A) Chickens were vaccinated with M2e-PP 3 times with 2 weeks intervals between the different doses (200, 50, 10, and 5 µg/bird) either via SQ with adjuvant or IN without adjuvant. IgG antibody responses were measured in the sera collected at 2 weeks after each immunization using free M2e peptide (left) or P particle (right) as coating antigen by ELISA. (B) Chickens were vaccinated with M2e-PP via SQ (5, 3 and 1 µg/bird) or transmucosal routes (IN, ED and MS) with a dose of 5 µg/bird. Antibody responses to M2e were measured by ELISA.
A. Serum antibody against M2e (top) and P particle (bottom) in Experiment (Continued)
Fig. 2.2. Continued

B. Serum antibody against M2e in Experiment 2
Figure 2.3. The protective efficacy of the 5 µg M2e-PP vaccinated SPF chickens in terms of reduction in virus shedding. Two weeks after the last vaccination, birds were challenged with low pathogenic H5N2 (A), H7N2 (B), and H6N2 (C) avian influenza viruses. Virus titers in tracheal and cloacal samples collected at 2 and 4 DPI were measured by quantitative real-time RT-PCR. Serum HI antibody titers were determined at 10 days after challenge (D). *p < 0.05 by ANOVA with Tukey–Kramer post hoc test.
A. Reduction in virus shedding against H5N2 virus challenge

B. Reduction in virus shedding against H7N2 virus challenge

(Continued)
C. Reduction in virus shedding against H6N2 virus challenge

D. Serum HI antibody titer at 10 days post challenge
Figure 2.4. The protective efficacy of a lower dose of M2e-PP (3 and 1μg/bird) vaccine via the SQ routes was compared to a 5μg/bird dose against H5N2 (A) and H7N2 (B) virus challenges. Serum HI antibody titers were determined using sera collected at 10 days after challenge (C). *p < 0.05 by ANOVA with Tukey–Kramer post hoc test.
A. Reduction in virus shedding against H5N2 virus challenge

B. Reduction in virus shedding against H7N2 virus challenge

(Continued)
C. Serum HI antibody titer at 10 days post challenge

![Bar chart showing serum HI antibody titer comparison between control and different groups at 10 days post challenge for H5N2 and H7N2 viruses.]
Table 2.1. Chicken immunization groups and challenge strains.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (µg)</th>
<th>Challenge strain</th>
<th>Total number of birds</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS control (Control)</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>P particle subcutaneous route with adjuvant (P particle)</td>
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<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>M2e-PP subcutaneous route (M2e-SQ)</td>
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<td>M2e-PP intranasal route (M2e-IN)</td>
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</tr>
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<td>M2e-PP Eye drop route (M2e-ED)</td>
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</tr>
<tr>
<td>M2e-PP via microsprayer (M2e-MS)</td>
<td>5</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* A/Chicken/PA/13609/93 (H5N2)

** A/Chicken/NJ/150383-7/02 (H7N2)

*** A/Chicken/CA/431/00 (H6N2)
2.7. References


Chapter 3: Supplementation of inactivated influenza vaccine with the norovirus P particle-M2e chimeric vaccine enhances the protection against heterologous virus challenge in chickens

3.1. Abstract

Current influenza vaccines provide satisfactory protection against homologous viruses with limited cross-protection against antigenically divergent strains. The highly conserved extracellular domain of the matrix protein 2 (M2e) has shown promising results as one of the components of a universal influenza vaccine in different animal models. As an approach to overcome the limited strain specific protection of inactivated influenza vaccine (IIV), a combination of recombinant M2e protein expressed on the surface of norovirus P particle (M2eP) with IIV was tested in chickens. Co-immunization of birds with both vaccines did not affect production of M2e-specific IgG antibody compared to the M2eP alone vaccinated group. However, the co-immunized birds showed significantly higher hemagglutination inhibition (HI) antibody titers against vaccine and challenge viruses as well as cross reactive antibody responses against different viruses (H5, H6, and H7 viruses) compared to the IIV alone vaccinated group. Upon intranasal homologous and heterologous virus challenges, combined vaccine groups showed greater reduction in viral titers from tracheal swabs compared to those
groups receiving IIV alone. Moreover, M2eP antisera from vaccinated birds were able to bind to their native target on both virus and infected cells as well as to inhibit viral replication. Our results support the potential benefit of supplementing IIV with M2eP to expand the vaccine cross protective efficacy.

3.2. Introduction

Avian influenza (AI) virus can cause devastating economic losses in poultry industry which include mortalities, reduced egg production and decreased weight gain. Moreover, it can cross the species barrier causing human infections, which has raised serious public health concerns (Perdue and Swayne, 2005). Vaccination remains the primary preventive measure against influenza viruses with induction of neutralizing antibodies to major viral proteins, particularly the hemagglutinin (HA) and to lesser extent neuraminidase (NA) (Kapczynski and Swayne, 2009). However, the increased incidence of diverse influenza A virus (IAV) infection in both animal and human populations worldwide and inability of conventional IIV to provide protection against new strains and subtypes emphasize the need to develop a universal or broadly reactive influenza vaccine that could protect against diverse IAV strains (Du et al., 2010; Ebrahimi et al., 2012; Wu et al., 2007a).

Recent studies showed that immunity against conserved influenza viral proteins (M2, HA2, M1, NP) can provide heterosubtypic protection and those proteins have been
targeted for the development of universal vaccine (Du et al., 2010; Pica and Palese, 2013). Influenza matrix protein 2 (M2), a small 97 amino acid (aa) protein, has an important role during virus replication by formation of ion channels in the viral membrane. Its pH regulatory function provides the un-coating of viral particles in endosomes and prevents premature conformational rearrangement of newly synthesized HA during transport to the cell surface (Schnell and Chou, 2008). Additionally, M2 forms a disulfide-linked homotetramer in its native form which is expressed on virions in low quantities, but abundantly presented on the surface of virus infected cells (Holsinger and Lamb, 1991; Hughey et al., 1995; Lamb et al., 1985; Zebedee and Lamb, 1988; Zebedee et al., 1985). The extracellular domain of M2 protein (M2e), a 24 aa protein, is highly conserved across different IAV subtypes and thus considered one of the candidate proteins for universal influenza vaccine development (Fiers et al., 2009; Neirynck et al., 1999). However, M2e protein is poorly immunogenic and efforts have been made to enhance its immunogenicity by fusing the M2e to appropriate carriers (Dabaghian et al., 2014; De Filette et al., 2008b; Ebrahimi and Tebianian, 2010; Xia et al., 2011), expressing through influenza virus-like particles (VLPs) (Kim et al., 2014b; Song et al., 2011b) and using different adjuvants such as Freund's adjuvant (Fan et al., 2004), monophosphoryl lipid A (Kim et al., 2014a), cholera toxin subunits (Liu et al., 2004a), and water-in-oil based adjuvants (Wu et al., 2009). These M2e constructs have induced a significant immune
response against M2e protein with variable degrees of protection in different animal models tested.

Despite the comprehensive reports of M2e-based vaccines, the full mechanism of cross protection induced by anti-M2e antibodies is not clear. It seems that M2e immunity confers their protection through non-neutralizing immune mechanisms, as M2e-specific antibodies do not directly neutralize viruses but they can inhibit viral replication (Zebedee and Lamb, 1988, 1989). Previous reports have shown the ability of mouse anti-M2e monoclonal antibody 14C2 to reduce either plaque sizes or growth rate (plaque number) of some IAV strains in vitro (Zebedee and Lamb, 1988). Moreover, passive immunotherapy with 14C2 monoclonal antibody reduced human influenza virus replication in the lung of mice (Treanor et al., 1990). Therefore, multiple mechanisms might be involved in conferring protection by M2e-specific antibodies. It was suggested that M2e-specific antibodies could disturb crucial interactions between the matrix protein 1 (M1) and M2 proteins and subsequently interfere with the interaction of the M1 protein with the HA, NA proteins, and the nucleocapsid complexes, thus interfering with virus assembly and causing growth restriction (Zebedee and Lamb, 1989). Moreover, anti-M2e antibodies bind to the virus-infected cells, proposing another indirect mechanism of protection. Non-neutralizing antibody-mediated protective mechanisms include natural killer cell antibody-dependent cellular cytotoxicity, complement-dependent cytolysis,
preventing the release of viral particles into the extracellular fluids, and/or enhancing the uptake by phagocytic cells (Jegerlehner et al., 2004).

Based on the aforementioned studies showing weak neutralizing ability, M2e-based vaccines would not be effective enough as a stand-alone universal vaccine to replace current IIV. Recent studies showed that supplementation of M2e antigen to the IIV could improve the limited cross protective efficacy of IIV. In mice, combination of M2e, either as VLP or recombinant protein, with IIV or split vaccine provided cross protection against lethal challenge with heterologous and heterosubtypic influenza viruses in terms of reducing weight loss, lung viral shedding titers and long-term cross protective immunity (Kim et al., 2014b; Lee et al., 2015; Song et al., 2011a). In chickens, supplementation of recombinant M2e protein (100 µg single dose) to IIV prepared from H9N2 virus (A/chicken/Korea/01310/2001) provided marginal additive effect in reducing heterologous H9N2 (A/Korean native chicken/Korea/K040110/2010) challenge viral titers from tracheal and cloacal swabs, tracheal tissues and cecal tonsils (Park et al., 2014). In another study, chickens vaccinated with IIV prepared from H5N1 virus, supplemented with M2e-5x VLPs were partially protected against lethal challenge with heterologous H5 highly pathogenic AI (HPAI) viruses (Song et al., 2016).

The Norovirus P particle has been proposed as a vaccine candidate for human norovirus and a good vaccine platform for antigen presentation (Tan et al., 2008a; Tan et al., 2011). Insertion of the AI virus consensus M2e sequence into loop 2 of the norovirus
P particle resulted in the formation of M2eP chimera. In our previous study, M2eP vaccine was highly immunogenic and provided partial protection against the challenge by 3 different subtypes of AI virus in specific-pathogen-free (SPF) chickens (Elaish et al., 2015). In this study, we hypothesized that supplementation of different H7 subtype IIVs with recombinant M2e protein presented on the surface of norovirus P particle will enhance the protective efficacy of HA based vaccines against homologous and heterologous AI viruses challenge in a chicken model. Our results indicate that M2eP supplementation to IIV has an adjuvant effect by significantly enhancing both the level of HI antibody against both vaccine and challenge virus and cross reactive antibodies recognizing different IAV subtypes and potentially broadening the cross protection of IIV. In addition, we demonstrated the ability of M2e-specific antibodies to bind to native M2e antigens expressed on the surface of whole virus particles and the infected cells plus their ability to inhibit viral replication \textit{in vitro}.

3.3. Material and Methods

3.3.1. Viruses and cells

The A/Chicken/NJ/150383-7/02 (H7N2), A/Turkey/OR/71 (H7N3), A/Chicken/PA/13609/93 (H5N2), and A/Chicken/CA/431/00 (H6N2) viruses were obtained from the repository of the Food Animal Health Research Program (Wooster, OH) and passaged once in 10-day-old SPF embryonated chicken eggs (ECEs) to prepare
working stocks for the study. Madin-Darby canine kidney (MDCK) cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 10 µg/ml gentamicin.

3.3.2. Preparation of M2eP, IIV and purified viruses

The M2eP chimeric protein containing the consensus M2e sequence of AI viruses was constructed, purified and characterized as previously described (Elaish et al., 2015).

The infectious allantoic fluid containing either A/Chicken/NJ/150383-7/02 (H7N2) or A/Turkey/OR/71 (H7N3) viruses was harvested and inactivated with 0.1% beta-propiolactone (BPL) (Sigma-Aldrich, Saint Louis, MO) as previously described (International Office of Epizootics., 2012). The IIV-H7N2 was used in homologous (Trial 1), while IIV-H7N3 was used in heterologous (Trials 2-1 and 2-2) virus challenge experiments.

For whole virus ELISA, the allantoic fluid was concentrated and purified by equilibrium density centrifugation through a 30% to 60% linear sucrose gradient as described previously (Tumpey et al., 2005).

3.3.3. Vaccination of birds

Three different trials have been performed. All the birds across different experiments were vaccinated via subcutaneous route (SQ) using Montanide ISA 70 adjuvant (Seppic, France) mixed in 3:7 volume ratios.
In Trial 1 (homologous virus challenge experiment), two-week-old SPF chickens were divided into 6 vaccination groups (12 birds/group). One group of birds was immunized with an emulsified solution of phosphate buffered saline (PBS) mixed with adjuvant and served as mock control (Mock), while another group was vaccinated twice with M2eP (5 μg / bird) with a two-week interval (M2eP-2x) (Table 3.1). In IIV groups, birds were vaccinated with IIV-H7N2 containing 256 hemagglutination unit (HAU) at either 2 (IIV-2w) or 4 weeks of age (IIV-4w). The combination vaccine groups were vaccinated either with IIV at 2 weeks followed by M2eP at 4 weeks of age (IIV, M2eP) or a mixture of IIV and M2eP at 4 weeks of age (IIV+M2eP).

In heterologous virus challenge trials, two experiments (Trials 2-1 and 2-2) were performed with some modification in the vaccination schedule and regime (Table 3.1). In Trial 2-1, vaccines and vaccination procedures for the first two groups (mock and M2eP-2x) was the same as Trial 1. For the IIV group, birds were vaccinated with IIV-H7N3 at 4 weeks of age (IIV-4w) containing 64 HAU, diluted 1/16 from the original stock containing 1024 HAU (Table 3.1). The birds in combination groups were vaccinated either with IIV combined with M2eP at 4 weeks of age (IIV+M2eP) or primed with M2eP at 2 weeks followed by combined vaccine at 4 weeks of age (M2eP, IIV+M2eP).

In Trial 2-2 (Table 3.1), compared to Trial 2-1, the M2eP group was vaccinated three times (M2eP-3x) instead of twice, the IIV group was vaccinated at 6 weeks of age instead of 4 weeks (IIV-6w) (using the same IIV dose, 64 HAU), and combination group was
primed with 2 doses of M2eP with two-week interval followed by combined vaccine at 6 weeks of age (M2eP-2x, IIV+M2eP). In order to assess the cross protective effect of M2e immunity and rule out the effect of HI neutralizing antibodies induced by IIV, birds in the combined vaccination groups showing similar HI titters (~ 3 and 5 log₂ in trial 2-1 and 2-2, respectively) against homologous vaccine virus to IIV alone groups were used for challenge.

3.3.4. **Viral challenge, sample collection, and quantification of viral RNA**

All vaccinated chickens were challenged through the intranasal route with 10⁶ EID₅₀/0.2 ml of A/Chicken/NJ/150383-7/02 (H7N2) two weeks after the last vaccination. Tracheal swabs were collected from the infected birds at the indicated time points, 3 and 5 days post challenge (DPC), and eluted in 1 ml of PBS supplemented with gentamicin (10 µg/ml) for virus detection. At 7 DPC, all birds were bled to determine the post-challenge HI titers (Pedersen, 2008).

The RNA was extracted from 100µl of the swab sample supernatant using a Viral RNA Extraction Kit® (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Viral genome amounts were determined by quantitative real-time RT-PCR (qRT-PCR) using influenza A virus matrix gene specific primers and probes as previously described (Lee and Suarez, 2004; Spackman et al., 2002). In order to allow interpolation of median egg infective dose (EID₅₀) titers of swab samples by the qRT-
PCR method, a standard curve was created by plotting cycle threshold (Ct) values generated with RNA extracted from serial 10-fold dilutions of the same virus stock (with known EID$_{50}$ titer) used to inoculate the chickens as a function of virus dilution. The curve was used to convert Ct values of tracheal swab viral RNA to EID$_{50}$ titers. EID$_{50}$ titers derived by qRT-PCR correlate well with the EID$_{50}$ titers measured in eggs (Lee and Suarez, 2004).

3.3.5. **Determination of M2e-specific IgG, whole virus specific antibody and HI antibody titers**

M2e-specific antibody responses were determined by ELISA using synthetic M2e peptide as a coating antigen (2 µg/ml, Ohio Peptide, Powell, OH) as previously described (Elaish et al., 2015; Xia et al., 2011).

Immune responses to the different virus strains were determined by ELISA using whole virus as the coating antigen as previously described (Quan et al., 2007) with modifications. Briefly, ninety-six well microtiter plates (Nunc MaxiSorp®, Thermo Fisher Scientific, Waltham, MA) were coated with 100 µl of purified viral antigens (4 µg/ml) at 4°C overnight. Next day, plates were incubated for 2 hours at room temperature (RT). The wells were then blocked with 200 µl of 5% nonfat dry milk in PBS and then incubated for 2 hours at RT. After washing six times, serially diluted sera (100 µl) in 2.5% nonfat dry milk in PBS were added to the coated plates for 2 hours at RT. Plates
were then washed six times with PBS (pH 7.4) and HRP-conjugated goat anti-chicken IgG (KPL, Gaithersburg, MD) was added. After incubating the plates for 2 hours at RT, the plates were washed six times followed by addition of the substrate, tetramethylbenzidine (TMB) substrate reagent set (KPL) (50 µL/well), and the plates were incubated for additional 15 min at RT. The reaction was stopped by adding 50 µl/well of 1M phosphoric acid, and the absorbance at 450 nm (A$_{450}$) was measured (i-Mark ELISA Reader, Bio-Rad, Life Science Research, CA, USA). Sera from mock vaccinated group were used as controls. Antigen-specific antibody titers were defined as the reciprocal of the highest dilution with an A$_{450}$ that is above the mean of the mock sera plus two standard deviations.

Two weeks after last vaccination, pre-challenge HI antibody titers (0 DPC) were determined using BPL-inactivated antigens, H5N2, H6N2, H7N2, and H7N3 viruses (H5N2-0 DPC, H6N2-0 DPC, H7N2-0 DPC, and H7N3-0 DPC). At 7 DPC, post-challenge HI antibody titers (H7N2-7 DPC) against H7N2 challenge virus were determined. The HI titers were determined using two-fold serially diluted serum samples after heat inactivation at 56° C for 30 min, 8 HAU of virus antigen and 1% turkey erythrocyte suspension as previously described (Pedersen, 2008).
3.3.6. MDCK cell ELISA

Sera were tested for reactivity with IAV infected MDCK cells to determine whether the induced antibody in immunized chickens recognizes native viral M2 as previously reported (Huleatt et al., 2008) with modifications. Briefly, MDCK cells were grown in 96-well culture plates in DMEM containing 10% FBS at 37°C till confluence. The growth media was removed and cells were washed 3 times with PBS. Half of the plate was incubated with 1 × 10^6 median tissue culture infectious dose 50% (TCID₅₀) per well of A/Chicken/NJ/150383-7/02 (H7N2), A/Chicken/PA/13609/93 (H5N2), or A/Chicken/CA/431/00 (H6N2) viruses (50 μl per well) in serum-free DMEM, and the other half plate was incubated with serum free DMEM. Following a 60 min incubation period at 37°C, 100 μl of DMEM complete medium with 10% FBS was added to each well and plates were incubated for 20 hours at 37°C. The next day, plates were washed three times with PBS and fixed with 10% formalin at RT for 10 min. Wells were washed three times with PBS and blocked with 200 μl of PBS + 5% FBS for 1 hour at RT. Selected M2eP-2x sera from Trials 1 and 2-1 (1: 40 dilution), showing the highest M2e-specific antibody titers, were serially diluted, added to the wells and incubated for 2 hours at RT. The wells were washed and incubated with HRP-conjugated goat anti-chicken IgG (KPL) for 2 hours at RT, followed by incubation with the TMB substrate reagent set (KPL) for a further 10 min at RT. The reaction was stopped with the addition of 50 μl of
1M phosphoric acid and the absorbance at 450 nm ($A_{450}$) was measured using an ELISA plate reader. Data reflect the mean $A_{450}$ ($\Delta A_{450}$: infected–uninfected cells) of triplicate wells per sample.

3.3.7. **Immunofluorescence assay (IFA)**

MDCK cells were seeded at $1 \times 10^6$ onto 24 well cell culture plates containing sterile glass cover slips and maintained by using DMEM complete medium with 10% FBS till confluence. Growth medium was then removed and cells was washed three times with PBS followed by infection with $1 \times 10^5$ TCID$_{50}$ of A/Chicken/NJ/150383-7/02 (H7N2) for 20 hours at 37°C. In the following day, the wells were washed three times with PBS, fixed in 4% paraformaldehyde (W/V) for 20 min at RT. The fixative was removed and cells were washed three times with PBS for 5 min each. Cells were then blocked with PBS+5% normal goat serum (Invitrogen, Rockford, IL) for 60 min at RT. After washing with PBS three times for 5 min each, the cover slips were removed from wells, incubated with chicken sera collected from M2eP-2x, IIV-H7N2, and mock vaccinated birds (1:300 dilution) for 2 hours at RT, followed by washing three times with PBS for 5 min each to remove unbound antibodies. The cells were stained with 1:500 dilution of Alexa Fluor 488 conjugated goat anti-chicken IgG antibody (Invitrogen, Rockford, IL) for 2h in dark at RT. After washing cover slips three times with PBS for 5 min each, it was counterstained with 1 µg/ml DAPI (Sigma-Aldrich, Saint Louis, MO).
for 5 min in dark. The cover slips were washed with PBS three times, mounted on glass slides. Fluorescence analysis and photography were performed on a Leica microscope (Leica Microsystems Inc., Buffalo Grove, IL) with 63 x magnifications.

3.3.8. Plaque reduction assay

MDCK cells were seeded at 2$x10^6$ onto a 6-well plate and allowed to grow till confluence. 100 plaque forming units of A/Chicken/NJ/150383-7/02 (H7N2) virus, diluted into DMEM containing 0.75 µg/ml TPCK-treated trypsin, was mixed (1:1) with serum dilutions being tested in micro-centrifuge tubes. The virus-serum mixture was further incubated for 60 min at 37°C for serum antibodies to bind the virus. In the meantime, the plates were washed three times with PBS. At the end of incubation, 200 µl of mixture (virus-antibody complexes) was added to each well and incubated in the CO₂ for additional 60 min at 37°C with intermittent rocking every 15 min. The plates were washed three times with DMEM and then overlaid with Minimum Essential Medium plus 1.2% agarose containing 0.75 µg/ml of TPCK-treated trypsin. The plates were incubated at 37°C for 3 days, fixed with 10% formalin, air-dried, and stained with 0.1% crystal violet to visualize and count the plaques. Each dilution, the cell control, and the virus control were tested in triplicate.
3.3.9. **Statistical analysis**

Data were analyzed using IBM SPSS Statistics (Version 22.0) software. To compare M2e-specific antibody, whole virus-specific antibody and HI antibody titers among IIV and M2eP-IIV combined groups, unpaired t-test was used. MDCK whole cell ELISA assay was evaluated using unpaired t-test comparing sera from M2eP vaccinated and mock control groups. While plaque reduction virus neutralization assays was evaluated using one-way analysis of variance (ANOVA) followed by the LSD post-hoc test comparing sera from M2eP vaccinated, mock control, and IIV-H7N2 vaccinated groups. Comparison of virus shedding titers among groups was made by ANOVA followed by the LSD post-hoc test. Statistical significance was determined as a p value <0.05.

3.4. **Results**

3.4.1. **M2e specific IgG, HI antibody and whole virus specific antibody responses**

M2e-specific IgG titer in the sera collected at 2 weeks after each immunization was determined (Fig. 3.1). Chickens vaccinated with M2eP via the SQ route developed detectable serum IgG levels after the first vaccination and the IgG antibody level increased after booster vaccination. In spite of the lower M2e-specific titers observed in Trial 2-2 compared to other trials, the similar trend of incremental increase in antibody titer after each booster vaccination was detected. Anti-M2e antibodies were not detected
in IIV alone groups in all three different experiments (Fig. 3.1). In Trial 1, supplemented vaccination of M2eP with IIV either as booster or simultaneous injection induced similar M2e antibody titers to that induced by a single dose of M2eP (Fig. 3.1A). The result was reproduced in Trials 2-1 and 2-2 where M2e-specific antibody titers induced by vaccination with IIV+M2eP were similar to those induced by a single dose of M2eP alone in non-combination group (Fig. 3.1B). Moreover, birds primed and boosted with M2eP followed by a combination of IIV-H7N3 and M2eP showed similar M2e antibody titers when compared to birds given 2 doses of M2eP, respectively (Fig. 3.1C). The antibody titers were slightly higher in IIV+M2eP combination groups compared with M2eP alone groups although the differences were not statistically significant. This higher trend of M2e specific titers in combination groups was also maintained in Trial 2-2 despite the overall lower titers observed in the whole experiment.

In Trial 1, pre-challenge HI antibody titers (H7N2-0 DPC) against the vaccine virus were ~2 fold higher in M2eP combined groups (“IIV, M2eP” and “IIV+M2eP”) than those detected in IIV alone although the values were not statistically significant (Fig. 3.2A). The boosting effect of M2eP was clearly demonstrated in Trials 2-1 and 2-2, where the groups M2eP was further supplemented as initial prime vaccination (i.e.: “M2eP, IIV+M2eP” and “M2eP-2x, IIV+M2eP”) showed significantly higher HI titers than IIV alone groups against homologous (H7N3) and heterologous (H7N2) viruses (Fig. 3.2B and C). In the groups primed with M2eP followed by combination vaccine, HI
titers were two and four fold higher against vaccine virus (H7N3) and challenge virus (H7N2), respectively, compared to the IIV alone group in Trial 2-1 (Fig. 3.2B). In Trial 2-2, pre-challenge HI titers were two folds higher against both vaccine virus (H7N3) and challenge virus (H7N2) than the IIV alone group (Fig. 3.2C). HI antibodies were not detected in the mock and M2eP groups at 0 DPC across different trials as expected. Birds vaccinated with either IIV alone or combined with M2eP showed low or no cross reactive HI antibody titers against H5N2 and H6N2 viruses in different trials (Fig. 3.2).

Using whole virus ELISA, in Trial 1, sera from “IIV, M2eP” and combined IIV+M2eP vaccinated group had significantly higher IgG antibodies (at least 2 folds) against heterosubtypic (H5N2 and H6N2) antigens compared with sera from birds vaccinated with IIV alone groups (Fig. 3.3A). Moreover, homologous virus specific (H7N2) IgG titers were statistically significant in birds vaccinated with combined IIV+M2eP, approximately 2 folds, compared with IIV alone group (IIV-4w). While “IIV, M2eP” group showed only a slight increment in IgG titers against H7N2 virus compared to IIV alone group (IIV-2w) which was not statistically significant. In Trial 2-1, the antibody response in the group primed with M2eP and boosted with IIV+M2eP was 2 folds higher than that detected in birds immunized with IIV+M2eP against homologous (H7N3) and heterosubtypic (H5N2 and H6N2) antigens. The IgG titers in this combined group was significantly higher than that detected in IIV alone group against H7N3, H5N2 and H6N2 viruses. Moreover, sera from “M2eP, IIIV+M2eP” vaccination group showed
statistically significant higher antibody titers against H7N2 challenge virus than IIV+M2eP or IIV alone group (~6 and 8 folds, respectively) (**Fig. 3.3B**). The results correlate with the enhanced protection against H7N2 virus challenge in M2e primed and combination booster group.

Immune sera from M2eP-2x vaccinated birds showed high levels of cross reactivity to the different virus subtypes tested (**Fig. 3.3**). Cross-reactivity with H7N2 virus was approximately four fold higher than that observed with either H5N2 or H6N2 viruses in different trials. The results indicate that M2eP are highly immunogenic and capable of inducing cross reactive antibodies to IAV regardless of HA subtype.

### 3.4.2. M2eP supplemented inactivated vaccine enhanced protection against homologous and heterologous virus challenges in SPF chickens

In Trial 1, homologous virus challenge experiment, birds vaccinated twice with M2eP were able to reduce the challenge virus shedding titers from tracheal swabs at both time points tested, 3 and 5 DPC (~20 and 4 folds, respectively), compared to the mock challenge group (**Fig. 3.4A**). Supplementation of M2eP to IIV, either as a booster dose or simultaneous immunization, showed ~ 4 and 3 logs lower virus titers compared to the mock challenge group at 3 and 5 DPC, respectively. Those combined vaccine groups showed a trend of lower virus titers compared to IIV alone vaccinated chickens, especially at 3 DPC but the differences were not statistically significant (**Fig. 3.4A**).
In the first heterologous virus challenge experiment (Trial 2-1), vaccination of birds with IIV-H7N3 alone showed slight reduction in shedding compared to control group (~3 and 40 folds reduction at 3 and 5 DPC, respectively) (Fig. 3.4B). Supplementation of IIV-H7N3 with one dose of M2eP (IIV+M2eP) showed further reduction in virus titers with no statistical significance compared to mock challenge group (~4 and 48 folds at 3 and 5 DPC, respectively). While, M2eP prime followed by combined IIV+M2eP (M2eP, IIV+M2eP) showed a significant reduction of challenge virus titers by approximately 40 and 60 folds compared to challenge control at 3 and 5 DPC, respectively. Remarkably, M2e priming were able to further reduce the viral titers by 10 folds especially at 3 DPC in comparison to IIV alone and IIV+M2eP groups with no differences between those 3 groups at 5 DPC. M2eP alone group did not show reduction in the challenge virus titers from tracheal swabs at both time points tested, 3 DPC and 5 DPC (Fig. 3.4B). From the aforementioned results, M2eP priming followed by combined vaccine booster was the most promising regime as shown by significant reduction of shedding titers especially at 3 DPC.

In Trial 2-2, we tested the effect of priming of birds with 2 doses of M2eP followed by combination with IIV and M2eP in providing better protection against challenge virus. As shown in Fig. 3.4C, the M2eP supplemented group had significantly lower viral titers by more than 1000-fold compared to the control group and by 10-folds compared to the IIV alone group at both 3 and 5 DPC. These results show that the
protective efficacy of M2eP priming and combination boosting was not only reproduced but also enhanced by boosting with a second dose of M2eP followed by combined vaccination. Birds vaccinated with M2eP only (M2eP-3x) did not show a statistically significant reduction in viral shedding titers at both 3 and 5 DPC, compared to the control (Fig. 3.4C).

To determine post-challenge HI titers as an indicator of active virus replication, we tested serum samples collected at 7DPC against H7N2 challenge virus (Fig. 3.5). At 7 DPC, the sera collected from both mock and M2eP groups, which supposed to have most active challenge virus replication, seroconverted and showed high post-challenge HI antibody titers (Fig. 3.5). In Trial 1, both IIV-2w and M2eP boosted (IIV, M2eP) groups did not show a significant increase in post-challenge HI titer indicating suppression of active virus replication in those vaccine groups (Fig. 3.5A). In other Trial 1 groups (IIV-4w and IIV+M2eP) and in heterologous challenge trials (Trial 2-1 and 2-2) where birds were vaccinated with reduced dose of H7N3 vaccine, increase in post-challenge HI titers correlated with lower protection from challenge virus. In all experiments, Post-challenge HI titers between IIV groups and M2e combined vaccination groups were not significantly different (Fig. 3.5).
3.4.3. **M2eP vaccine-derived antibodies recognize their native epitopes on influenza virus infected cells**

Selected M2eP serum samples from vaccinated birds in Trial 1 and 2-1 were used to determine their ability to recognize the native M2 protein expressed on virus infected cells by whole cell ELISA. As shown in Fig. 3.6, sera from vaccinated bird recognized and bound similarly to M2e expressed on MDCK cells infected with three different AI viruses. Serial dilutions of tested sera displayed the gradual reduction in reactivity to the virus infected cells with an endpoint titer of 1:320. To the best of our knowledge, this is the first report of M2e vaccine induced antibody binding to its native M2e epitopes on the surface of virus infected cells in a chicken model.

Using fluorescence microscopy, the binding of anti-M2e antibody (M2eP-2x immunized chicken sera) to M2e protein on the surface of infected cells was demonstrated (Fig. 3.7). The fluorescence intensity was comparable between anti-M2e antisera and anti-H7N2 antisera (Fig 3.7B and C). No fluorescence was observed with mock sera as expected (Fig. 3.7A).

3.4.4. **In vitro neutralization by M2e-specific antibodies**

Whole cell ELISA and IFA results have shown the ability of M2eP induced antibodies to recognize native M2e on infected cells. Plaque reduction assay was conducted to determine if antibodies induced by M2eP vaccination can inhibit virus
replication. Selected serum from M2eP-2x vaccinated birds (Trial 1) was tested at 5 different dilutions (100 to 1000000) alongside with sera from mock and IIV-H7N2 vaccinated birds (Fig. 3.8). Although the mock control serum showed minor nonspecific inhibitory effect, M2eP serum demonstrated significant reduction in plaque formation. The maximum neutralization index of M2eP serum was approximately 40%. As expected, the H7N2 sera showed strong neutralizing activity in all dilutions tested compared to M2eP sera. M2eP-2x and IIV sera gradually displayed the reduction in neutralization ability with higher dilutions of sera which was not obvious at 100 and 1000 dilutions.

3.5. Discussion

The efficacy of several M2e-based vaccines has been tested in different animal models and demonstrated substantially different results depending on the animal model used, vaccine construct and other experimental conditions. In mice, vaccination with M2e alone confers protection against different subtypes of influenza viruses, including HPAI H5N1 virus. However, the protective efficacy of M2e vaccines in chickens is generally much lower than that observed in mice despite the induction of high M2e specific antibodies (Dabaghian et al., 2014; De Filette et al., 2006a; Elaish et al., 2015; Layton et al., 2009; Xia et al., 2011; Zhang et al., 2011). More importantly, M2e vaccine, when used alone, is less efficacious than oil-adjuvanted IIV in terms of reducing the level of viral shedding against the homologous virus challenge (Swayne et al., 1999). Therefore, it is unlikely that M2e-based vaccines could be developed as standalone vaccines that
would completely replace the current HA based influenza vaccines in chickens. Accordingly, this study was conducted to determine the efficacy of IIV supplemented with recombinant M2e protein against homologous and heterologous AI viruses challenge in chickens.

As observed in our previous study with 3 vaccinations of M2eP (Elaish et al., 2015), a prime-boost vaccination with two doses of M2eP (5 µg/bird) via SQ route was sufficient to induce high levels of M2e specific IgG antibodies in chickens (Fig. 3.1). Additionally, the levels of M2e specific antibody titers in all combined vaccine groups were similar to those observed in groups using M2eP alone, indicating that immune interference did not occur when M2eP and IIV were used simultaneously. When used alone, vaccination with IIV did not induce detectable levels of M2e-specific antibodies (Fig. 3.1) which is consistent with the previous studies indicating that neither vaccination nor infection will induce significant anti-M2e antibodies (Jegerlehner et al., 2004; Wu et al., 2007a). The low immunogenicity of M2e in the context of IIV or live virus may due to the shielding effects by HA and NA proteins which might prevent the access of immune effector cells to M2e protein.

While combined vaccination did not affect induction of M2e-specific antibodies as evidenced by similar M2e titers observed among vaccinated groups, it significantly boosted induction of HI antibodies that were cross reactive with both homologous and heterologous viruses (Fig. 3.2). These results indicate the potential adjuvant effect of the
carrier norovirus P particle on immune response induced by IIV. The norovirus P particle is an efficient carrier and antigen presenter that converts non or weakly immunogenic antigens into highly potent immunogen with induction of detectable immune response as evidenced by presentation of M2e protein of influenza virus, inducing B cell proliferation and antibody synthesis against both P particle and antigen presented in their context (Tan et al., 2011; Tan and Jiang, 2012). To our knowledge, the current study is the first to report enhancement of HI antibody response by M2e-based vaccine in chicken or food animal model.

The native M2 protein forms a tetramer harboring its structural epitopes and recombinant M2e vaccines capable of inducing antibodies which target native M2 and bind to the surface of influenza infected cells may confer protection (Zebedee and Lamb, 1988). In addition, influenza virus-infected cells express high levels of M2 on the cell membrane and can be used to evaluate binding of M2e-based vaccine induced antibodies to their targets on cells. In this study, sera from M2eP vaccinated birds showed reactivity to cells infected with 3 different viruses as shown by MDCK whole cell ELISA, which suggests that M2eP specific antibodies would efficiently bind to M2e protein expressed on virus infected cells regardless of virus HA subtype (Fig. 3.6). Our data is comparable to previous reports in mice (Huleatt et al., 2008; In-Soo et al., 2013; Stepanova et al., 2015) although the endpoint titers in the current study were lower. The difference in titer may be attributed to the antibody titers induced after vaccination and the different
constructs used in different animal models (i.e. chicken vs mice). Furthermore, the recognition of anti-M2e antibody (M2eP-2x immune sera) was confirmed by IFA (Fig. 3.7) in agreement with the previous studies (Ernst et al., 2006; Zhang et al., 2011).

In our combination vaccine groups, we observed that supplementation of IIV with M2eP resulted in enhanced levels of antibodies that recognize M2e and are cross reactive with IAV of different subtypes (Fig. 3.3). The finding of the present study is consistent with previous reports in both mice and chickens (Kim et al., 2014b; Song et al., 2016). Moreover, M2eP-2x vaccinated sera showed cross reactivity to 3 different AI viruses as shown by whole virus ELISA (Fig. 3.3). The endpoint titers against H7N2 virus was approximately four fold higher than that observed against H5N2 and H6N2 viruses which could be attributed to the amino acid difference between the sequence presented on norovirus P particle and tested viruses (100% identity to H7N2 and one amino acid difference with H5N2 (C17R) and H6N2 (D21G) viruses, respectively). A study in mice showed that the endpoint titers detected after vaccination with M2e5x VLP against H3N2 virus was approximately four fold higher than that observed against H1N1 and H5N1 viruses which showed similar binding properties to M2e5x VLP immune sera (Kim et al., 2013b). As in our study, the amino acid difference between the sequence presented on the construct and that of tested viruses could have affected the results. Previous reports have indicated that the N-terminus, the first 12 amino acids of M2 protein, may contain one epitope that can induce antibodies with inhibitory activities against IAV replication in
vitro, while the other parts of M2e do not seem to have the ability to induce such kind of antibodies and do not abrogate the binding to native M2 protein (Huleatt et al., 2008; Liu et al., 2004b; Zou et al., 2005). Additionally, proline at residue 10, isoleucine at residue 11 and glutamic acid at residue 14 were found to be the most important for antibody binding. Thus, the cross reactivity conferred by M2eP is crucial for broader protection and the importance of minor M2e sequence difference on antibody recognition should be further investigated. Additionally, M2eP vaccination played an important role in improving the HI titers against both homologous and heterologous viruses which was not previously observed in chicken study (Song et al., 2016). Taken together, the aforementioned results emphasize the potential benefit of M2e supplementation of M2eP in boosting the immunogenicity and cross-reactivity of the IIV with different influenza virus subtypes.

Previous reports on the effect of anti-M2e antibody on influenza virus replication are still controversial. Zebedee and Lamb (1988) reported that 14C2 M2e-specific monoclonal antibody have the ability to either reduce the plaque size or the rate of growth and the number of plaques of certain influenza strains. In contrast, the humanized single chain Fv 14C2 antibody reduced not only plaque size but also the number of plaques for the same strain (Gabbard et al., 2009). The calculated neutralization ability of this antibody was approximately 30-40 % in both studies. In the current study, we demonstrated that polyclonal M2e serum from chicken could reduce the plaque formation
of influenza virus. The gradual reduction of this inhibitory function was not observed at the initial lower dilutions of M2eP sera which might be attributed to partial effect of M2e-specific antibodies in inhibiting the virus replication. While further dilution of sera showed gradual loss of activity. Moreover, we observed minimal non-specific inhibitory effect of mock sera as previously reported (Ryan-Poirier and Kawaoka, 1991) which may due to the presence of non-specific inhibitors in the chicken sera. Consistent with the results obtained from the 14C2 monoclonal antibody, polyclonal M2e serum could partially inhibit up to 40% of the virus. On the contrary, a previous study in chicken have shown the inability of M2e vaccine sera to neutralize or reduce the plaques of an H5N2 virus (Babapoor et al., 2011). The difference between the two studies might be attributed to the nature of M2e specific antibody or sensitivity of different viral strains used in the studies. Zebedee and lamb (1988) have found that the sensitivity of 14C2 antibody was dependent on viral strain tested. Despite the M2e sequence similarity, changes in other domains of M2 protein may govern antibody restricted growth. They noted the 3 residues difference in the cytoplasmic domain between antibody sensitive and resistant strains that correlated with the inhibitory effect of 14C2 antibody. Further studies are needed to investigate the effect of amino acid substitutions and the sensitivity of M2eP induced antibodies against different viral strains and subtypes.
In Trial 1, M2eP vaccination were able to reduce the H7N2 virus shedding in tracheal swabs by approximately 20 folds compared to the mock vaccination group. However, we were not able to clearly demonstrate the additive effect of M2eP in combined vaccination groups which may due to the use of a high dose of IIV and challenge with homologous virus. The combined vaccine groups showed a trend of enhanced reduction in virus shedding especially at 3 DPC, the peak point of virus replication, compared to their counter parts of IIV alone groups (Fig. 3.4A). In the heterologous virus challenge experiments (Trials 2-1 and 2-2), M2eP vaccination did not provide the expected reduction of shedding titers after H7N2 virus challenge (Fig. 3.4B and C). We demonstrated that anti-M2e antibodies are capable of binding to the M2 proteins that are abundantly presented on the surface of the infected cells (Fig. 3.6B). However, they can only induce a weak neutralization ability compared to HA protein induced antibodies (Fig. 3.8) which could describe the observed variability in protection. In addition, the role and involvement of natural killer cell might have led to an efficient delay in challenge virus clearance in M2e vaccinated chickens as previously described (Jegerlehner et al., 2004), which require further studies on mechanism of protection of M2e based vaccines in chickens. While in Trial 2-2, lower protection with M2eP-3x group correlated with the low M2e antibody titers detected. Adjustment of the vaccine volume to match up the IIV alone or combined groups had led to overall reduction of anti-M2e antibodies despite three times of vaccination. On the other hand, in Trial 2-1
and 2-2 where diluted dose of IIV was used, priming the chickens with M2e, once or twice, followed by the combined vaccination was able to significantly reduce the challenge virus shedding titers compared to IIV alone group indicating that M2eP played an important supplementary role in improving the IIV protection. A similar observation was reported by Park et al. (2014) where chickens were vaccinated with an H9N2 IIV supplemented with recombinant protein containing three tandem copies of M2e and then challenged with a heterologous H9N2 virus. It is worthy to note that birds in the combined vaccine groups showing HI titer similar to IIV alone group were selected in order to examine the cross protective potential provided by the M2e immunity, not by the neutralizing HI antibody. Hence, we strongly believe that the M2eP supplemented IIV groups could have shown more profound protection compared to IIV alone groups if the birds with high HI titers were included. Taken together, supplementation of IIV with M2eP can broaden the protective efficacy of IIV by inducing subtype independent M2e immunity and also by enhancing the cross-neutralizing HI and whole virus antibody titers. Future studies are warranted to further optimize the combination vaccine regimes and to better understand the mechanism of protection conferred by M2eP vaccination in chickens in comparison to mice in order to explain the difference in protection and to assess the usefulness of the different animal models in influenza vaccine studies.
3.6. Acknowledgements

The authors would like to thank Megan Strother for technical assistance and Dr. Tea Meulia for her help with confocal microscope. This study was supported by USDA, AFRI grant (2012-04042) and funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Mohamed Elaish is supported by a PhD scholarship from the Egyptian Ministry of Higher Education.
Figure 3.1. M2e specific antibody titers observed in three different experiments. Chickens were vaccinated at 2 and 4 weeks of age (A and B), and 2, 4, and 6 weeks of age (C) with different vaccination regimes. Two weeks after each vaccination, sera were collected to determine M2e-specific IgG antibody titers by ELISA using M2e synthetic peptide as a coating antigen. Data are expressed as mean ± standard error of mean.
A. Trial 1

B. Trial 2-1

C. Trial 2-2
Figure 3.2. Pre-challenge HI antibody titers induced in vaccinated chickens. Sera were collected two weeks after last vaccination. Pre-challenge antibody titers were determined using H7N2 and H7N3 vaccine viruses (H7N2 - 0 DPC and H7N3 - 0 DPC). Statistical significance between pre-challenge antibody titers of IIV group and M2eP combination groups was determined with unpaired t-test (**p < 0.01, ***p < 0.01). Data are expressed as mean ± standard error of mean.
Figure 3.3. Whole virus ELISA using 3 different influenza viruses. Antibody titers were determined using purified inactivated influenza viruses (H5N2, H6N2, H7N2, and H7N3) two weeks after vaccination. Results are expressed as the mean ± standard error of mean. Asterisk indicates significant differences between sera of IIV and IIV combined with M2eP (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).
Figure 3.4. Protective efficacy of different vaccines and vaccination regimes. The EID$_{50}$ equivalent virus titers in tracheal swabs collected at 3 and 5 days post-challenge (DPC) were interpolated from qRT-PCR Ct values as described in Materials and Methods. Statistical significance between mean titers was determined with ANOVA followed by LSD post-hoc test using SPSS ($^*p<0.05$, $^{***}p<0.001$). Data are expressed as mean ± standard error of mean. EID$_{50}$ median egg infectious doses.
A. Trial 1

B. Trial 2-1

C. Trial 2-2
Figure 3.5. Post-challenge HI antibody titers in vaccinated chickens. Sera were collected 7 DPC and tested using H7N2 challenge virus (H7N2 - 7 DPC). Data are expressed as mean ± standard error of mean.
Figure 3.6. Whole virus ELISA using influenza virus infected MDCK cells.

Antibody titers were determined using cells infected with H5N2, H6N2, and H7N2 two weeks after PBS (mock) or M2eP booster (M2eP-2x) vaccination. Data represent the ΔA₄₅₀ (infected minus uninfected cells) from five vaccinated chickens.
Figure 3.7. Immunofluorescence staining of influenza infected cells. MDCK cells were infected with $10^5$ TCID$_{50}$ of A/Chicken/NJ/150383-7/02 (H7N2) overnight and fixed with 4% paraformaldehyde. The infected cells were then incubated with PBS (mock), M2eP-2x (anti-M2e), and H7N2 (anti-H7N2) sera, then stained with Alexa flour 488 goat anti-chicken IgG antibody and counterstained with DAPI. The photos were acquired at 63x magnification power. Scale bars are 30 µm.
A. Mock

B. M2eP-2x

C. IIIV-H7N2
Figure 3.8. Plaque reduction assay to determine neutralizing capability of sera obtained from chickens vaccinated with PBS (mock), M2eP (anti-M2e) or IIV-H7N2 (anti-H7N2). Statistical significance between mean plaques was determined with ANOVA followed by LSD post-hoc test using SPSS (*p < 0.05, **p < 0.001). Data are expressed as mean reduction % ± standard error of mean. No plaques were seen in uninfected cells (negative control).
Table 3.1. Vaccination groups and schedule for 3 experiments

**Trial 1**

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**Trial 2-1**

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3.7. References


overcomes strain-specific protection of split vaccine against influenza A virus. Antiviral Res 122, 82-90.


Chapter 4: Summary and Future direction

AI, a primarily a disease of avian species, is caused by different subtypes of IAV. AI has the potential to cause devastating effects to the poultry industry. Additionally, some outbreaks in poultry have been associated with human transmission. The World Health Organization considers the AI a public health risk with pandemic potential and recommends that all nations develop a national influenza preparedness plan. Thus, control and prevention of the disease in poultry is critical and the most effective way is by vaccination.

Vaccines against AI are important tools for protecting the poultry industry and humans, since vaccines help increase resistance to infection, prevent illness and death, reduce virus replication and reduce viral transmission to birds and mammals, including humans. Accordingly, a wide variety of vaccines against AIV has been developed and tested in experimental conditions. However, current vaccines are mainly effective against matching vaccine strain but fail to induce cross-protective immune response against constantly emerging antigenic variants of IAV. This call for the development of a universal influenza vaccine, which can confer protection against a broad spectrum of influenza viruses. Currently, conserved IAV proteins, like the extracellular domain of Matrix 2 protein (M2e), have drawn general interest as targets for vaccine development.
However, M2e suffers from its inherent low immunogenicity and several approaches have employed to enhance anti-M2e immune responses in vaccinated animals.

In the present study, an avian M2e consensus sequence expressed on the surface of norovirus P particle (M2e-PP) was examined for its immunogenicity and protective efficacy against challenge with 3 different LPAI (H5, H6, and H7) in chicken (chapter 2). Chapter 3 describes the combined vaccine approach where a combination of M2e-PP with different H7 subtype IIV was investigated to determine the additive effect of M2e-PP on broadening the protective efficacy of IIV against both homologous and heterologous challenge. Finally, the ability of M2e-PP induced antibodies to bind to their native targets expressed on IAV and their \textit{in vitro} ability to reduce the plaque formation of IAV was examined.

In chapter 2, our first goal was determination the appropriate vaccination regime for M2e-PP in chicken including dose, route and schedule. In that regard, two weeks old SPF chickens was vaccinated three times with M2e-PP either via SQ with Montanide ISA 70 adjuvant or IN without adjuvant routes in two week interval using four different doses (200, 50, 10, and 5 µg/bird). The PP only was also included in that experiment with a dose of 10 µg/bird via the same routes (i.e. SQ and IN). The anti-M2e immune response was monitored by determining the M2e-specific IgG titer in the sera collected at 2 weeks after each immunization. Chickens vaccinated with M2e-PP via the SQ route with adjuvant developed detectable serum IgG levels after the first vaccination which was
increased after each booster vaccination. The ELISA endpoint titer showed that the lower doses used in this experiment (i.e., 5 and 10 µg/bird) induced a higher level of M2e antibody when compared to the two higher doses (50 and 200 µg groups). For the IN groups, we were not able to detect IgG or IgA in any of the vaccinated birds except for a minimal IgG titer observed in birds vaccinated with the 200 µg dose after the third vaccination. Based on the result obtained from the first experiment, two additional doses (1 and 3 µg/bird) of M2e-PP were tested in comparison to the 5 µg/bird dose (lowest dose used in previous trial). We also included ED and MS groups in addition to IN group, transmucosal routes, to enhance mucosal immunity against M2e. The M2e antibody response after each vaccination showed an increase in IgG titer by M2e-PP boosting as observed in the first experiment. In addition, both of the two lower dose groups (1 and 3 µg /bird) showed comparable serum IgG titers to that of the 5 µg/bird dose. Consistent with our first experiment, neither IgG in the sera nor IgA in the nasal washes of transmucosal groups was detected at all time points tested. The protective efficacy and the spectrum of the specific M2e antibody responses induced by M2e-PP chimera were evaluated by the level of virus shedding reduction after challenge with LPAI strains. Two-weeks after the third vaccination, birds were challenged with H5, H6, and H7 LPAI strains. Overall, chickens vaccinated with the 5 µg dose of M2e-PP via the SQ route with adjuvant showed a significant reduction in virus shedding after challenge with H5N2, H7N2, and H6N2 viruses. Despite the absence of detectable IgG and IgA responses in
birds vaccinated via IN routes, a similar level of reduction in virus shedding was observed in the IN group compared to the SQ group. The reduction of virus shedding in M2e-PP via SQ and IN vaccinated groups was more significant in both H5N2 and H6N2 challenged groups. In addition, the reduction was more statistically significant from the control group as compared H7N2 challenged groups. Although the low doses (i.e., 3 and 1 µg /bird) of vaccine induced comparable IgG titers to those obtained by vaccinating birds with 5ug/bird, they also provided similar protection against H5N2 and H7N2 virus challenges as observed with the 5 µg dose with less frequently observed statistical significance. In conclusion, The M2e-PP chimeras were immunogenic when administered via the SQ route with adjuvant and could provide protective antibody responses in chickens against different AI virus subtypes. The protection observed in the IN immunized group (despite the absence of IgG titers) and the differences between the protections of 1&3ug SQ groups as compared to the 5ug SQ (despite the presence of comparable IgG titers), may indicate that the protection mechanism provided by M2e vaccination is not only dependent on the M2e specific IgG titers and another mechanism is involved that need to be investigated.

Considering the limited protective efficacy of IIV especially against antigenically divergent heterologous strain, supplement vaccination with M2e was examined against homologous and heterologous AI viruses challenge in chickens (chapter 3). For that
purpose, three different experiments were performed where M2e-PP was combined with IIV prepared from different H7 subtype viruses. M2e specific ELISA results indicated that chickens vaccinated with M2eP via the SQ route developed detectable serum IgG levels after the first vaccination which increased after booster vaccination which is consistent with previous study. Moreover, supplemented vaccination groups with M2e-PP and IIV induced similar M2e-specific antibody titers to that induced by M2e-PP alone groups. On the contrary, anti-M2e antibodies were not detected in IIV alone groups across different experiments. Additionally, we tested the vaccine virus specific antibodies by HI assay and whole virus ELISA. M2e-PP combination groups with IIV showed an enhanced cross-neutralizing HI and whole virus antibody titers against vaccine, challenge, and heterosubtypic virus when compared to IIV alone groups. Upon challenge with homologous virus, supplementation of M2e-PP to IIV, either as a booster dose or simultaneous immunization, showed a trend of lower virus titers compared to IIV alone vaccinated chickens, especially at 3 DPC. In the first heterologous challenge experiment, vaccination of birds with IIV alone showed slight reduction in shedding compared to control group. While, M2e-PP prime followed by combined IIV+M2eP showed a significant reduction of challenge virus titers compared to challenge control at different time points tested. Remarkably, M2e priming were able to further reduce the viral titers by additional 10 folds especially at 3 DPC in comparison to IIV alone and IIV+M2eP groups with no differences between those 3 groups at 5 DPC. In the second heterologous
challenge experiment, the protective efficacy of M2e-PP priming and combination boosting was not only reproduced but also enhanced by boosting with a second dose of M2e-PP followed by combined vaccination which indicates that M2e-PP priming followed by combined vaccine booster was the most promising regime as shown by significant reduction of shedding titers. Additionally, whole MDCK cell based ELISA, IFA and whole virus ELISA showed the ability of M2e-specific antibodies to recognize their native M2e epitopes expressed on virus infected cells and whole virus. Finally, M2e-PP vaccine induced antibodies demonstrated significant reduction in plaque formation with a maximum neutralization index of approximately 40%.

The overall conclusion of this study was that M2e-PP is highly immunogenic in chickens via SQ with a dose as low as 1µg/ bird. When used alone it was able to partially protect chickens against the challenge with 3 different LPAI. In combination with IIV, M2e-PP supplementation enhanced HI antibody titers and cross neutralizing antibodies recognizing different viruses and a potential in broadening the cross protection of IIV. In addition, M2e-PP are capable of inducing cross reactive antibodies to IAV regardless of HA subtype that are able to recognize the native antigens expressed on the surface of infected cells. Those antibodies are able to inhibit viral replication in vitro.

Future studies are warranted to identify the immune mechanism of protection by M2e specific antibodies that will allow identification and validation of correlates of protection in chickens in comparison to mice in order to explain the difference in
protection and to assess the usefulness of the different animal models in influenza vaccine studies. Additionally, the need for further improvement of the vaccine in order to induce stronger humoral response and broaden immune protection by M2e. This may be achieved by maximum utilization of 3 surface loops of norovirus P particle to present multiple copies of M2e and finally, increasing the epitope density from 24 to 72 M2e copies. Moreover, presenting multiple M2e copies covering various sequences from different strains can help to broaden the immune protection of M2e. This was previously reported by M2e VLP presenting sequences from avian, human and swine viruses that conferred broadened protection in mice.

M2e-specific antibodies, mainly IgG, are the main actors in immune protection and it is also well documented that mucosal immunization with M2e-based vaccines offers better protection in mouse model compared to parenteral immunization strategies. This improved protection may be attributable to the induction of M2e-specific IgA. From our studies, mucosal immunization with M2e-PP failed to induce IgG or IgA antibodies which indicated the need of strong adjuvant to induce M2e immune response at the ports of entry of IAV. Co-administration of adjuvants is an effective approach for inducing cross-protection. Toll-like receptors (TLRs) were proven to be highly effective in inducing potent immune response. For example, flagellin, which is a TLR-5 agonist, has been used in several studies and shown to enhance immune responses and cross-protection when used as an adjuvant in VLP expressing influenza HA or when it is fused
to HA or M2e. In a chicken study, vaccination of IIV either intramuscularly or intranasally along with TLR5 ligand enhanced IAV specific IgA antibody titers suggesting its use against pathogens that invade through mucosal tissues. Other TLR ligands such as poly I:C and CpG (TLR3 and TLR21 ligands, respectively) have also been found to enhance the host’s immunity against the AI, and in this context, Intranasal immunization with H5N1 and either ligand resulted in the production of IL-12, IFN-γ and IL-6 in the nasal cavity and trachea, indicating induction of cellular immune response and induced significant levels of secretory IgA in respiratory tract and anti-AI-specific IgG in the serum. Thus, different combinations of TLR agonists aimed at selectively enhancing different cytokines could be incorporated in the vaccine to augment antibody responses and to curtail virus shedding leading to augmentation of heterosubtypic protection in chickens.

From our combination vaccination studies, M2e based vaccines will likely not be a complete substitute for the current influenza vaccines that are able to confer much stronger protection but against a very narrow antigenic range of viruses. In the future, with many other universal influenza vaccine candidates on the horizon, M2e-conjugate vaccines will likely find a place as part of a vaccine that is a blend of different conserved epitopes that together may offer strong, long lasting, and foremost broad immune protection. Another target for universal vaccine approach is HA2 subunit. The influenza virus HA2 subunit is more conserved than the HA1 subunit. Consequently, immune...
responses induced by the HA2 subunit are expected to elicit broad cross-protective antibodies against divergent strains from different influenza virus groups, suggesting its development as a universal vaccine. Studies have shown that vaccination with a synthetic HA2 peptide protected mice from H1N1, H3N2 and H5N1 influenza virus challenge. A recent study have reported the protective efficacy of M2e-HA2 fusion peptide on a mouse model. Use of M2e-HA2 fusion peptide enhanced protection against homologous and heterologous challenge compared to each peptide alone. M2e-specific antibodies have shown limited neutralization ability and indirect mechanisms to prevent IAV infection. While HA2 specific antibodies showed neutralizing properties against different IAV. Thus, combination of these two peptides can increase synergetic effects on each other’s immunogenicity and neutralizing properties. Taken together, considering the conserved nature of M2e and HA2 sequences and different mechanism of protection induced by the use of the single epitopes (M2e or HA2), it seems that the combination of both proteins together could improve the universal vaccine approach. Thus, this approach expands the efficacy of the vaccine by inducing cross protective immune responses (humoral & CMI) against divergent IAV subtypes, reduces the time and cost of manufacture compared to processes that target single peptides, and finally obviate the need for an annually reformulated vaccine and substantially enhance our pandemic preparedness of influenza vaccine for both human and animals.
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