2. Review of literature

2.1 Influenza viruses

Influenza virus is a classic example of an antigenically variable pathogen. Antigenically variable pathogens cause much of the burden of infectious diseases today because these pathogens can escape from the immunity induced by either vaccination or previous infection (Smith et al., 2004). The segmented nature of the virus facilitates an exchange of the entire genes among different influenza viruses during co-infection in a host resulting in new strains with pandemic potential. These pandemic viruses later establish themselves as seasonal influenza viruses and cause annual epidemics (Westgeest et al., 2014).

Global influenza surveillance data indicate that every month somewhere in the world influenza viruses are isolated from humans. Influenza virus activity peaks during the winter months in temperate regions. In the Northern Hemisphere, influenza virus activity typically occurs between November and March, whereas in the Southern Hemisphere it occurs between April and September. However, influenza can occur throughout the year in the tropical regions. During average epidemics, overall attack rates of influenza viruses are estimated to be 10–20%, but higher attack rate of about 40-50% can occur in certain susceptible groups such as schoolchildren, elderly populations or nursing home residents (Cox & Subbarao, 2000).

Epidemics of influenza are mainly attributed to drift in the HA and the NA proteins that circumvent adequate pre-existing immunity from vaccination or natural infection to render the individual susceptible to infection (Lofgren et al., 2007).

2.2 Neuraminidase (NA) protein: structure and function

NA is a mushroom shaped tetrameric transmembrane glycoprotein made of four identical subunits. It is the second major surface glycoprotein present in influenza A and B viruses, but absent in C viruses (Knipe & Howley, 2013). NA protein is made of four main regions: a cytoplasmic tail domain (six amino acids), a hydrophobic transmembrane domain (7-29 amino acids), a stalk (~50 amino acids), and a globular head containing the enzyme active site (19
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amino acids) (Figure 2; Air, 2012). The structure of each NA subunit (~469 amino acids) consists of six topologically identical 4-stranded anti-parallel \( \beta \) sheets arranged like the blades of a propeller (Figure 3). The enzyme active site (19 amino acids) is located at the centre of each subunit. The active site is a deep pocket made of amino acids that remain conserved in all the strains of influenza. Antibody binding sites are located on the surface loops that surround the enzyme active site (Varghese et al., 1983; Colman, 1994).

![Image of influenza neuraminidase sequence]

**Figure 2: Sequence of influenza neuraminidase**

Amino acids that form the cytoplasmic tail, transmembrane domain, stalk and globular head are shown. Secondary structures based on PDB ID: 4B7Q is shown and six propeller \( \beta \) sheets (\( \rightarrow \)) are coloured differently. Helices (\( \nearrow \)) and loops (\( \searrow \)) are also shown. The six 4-stranded anti-parallel \( \beta \) sheets arranged like the blades of a propeller are marked and coloured in magenta, red, green, yellow, blue and orange. Drug binding sites are highlighted and coloured in blue.
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The function of NA is to cleave the terminal sialic acid residues present on cell surfaces and progeny virions, facilitating the release of virus from infected cells and thus playing an important role in both the release and the spread of progeny virions. Some studies have also demonstrated NA's role in facilitating hemagglutinin-mediated fusion and virus invasion in the ciliated epithelium of human airways (Matrosovich et al., 2004).

![Image of three-dimensional structure of influenza neuraminidase protein]

**Figure 3: Three-dimensional (3D) structure of influenza neuraminidase protein**

The 3D structure of NA protein is depicted as a cartoon. The six 4 stranded antiparallel β sheets in each tetrameric subunit are shown and coloured like in Figure 2. Helices and loops are coloured in cyan. Oseltamivir is shown as sticks in one of the monomer catalytic site. Protein Data Bank (PDB) ID: 3T16 [www.pdb.org](http://www.pdb.org) was used for generating figures (Vavricka et al., 2011). The figure is rendered using PyMOL [www.pymol.org](http://www.pymol.org).
2.3 Neuraminidase inhibitors

The emergence of resistance to adamantane group of influenza antivirals targeting the M2 gene diverted the research interest towards the NA inhibitors, which later surfaced as the next important class of antivirals for the treatment and prevention of influenza (www.cdc.gov/flu/professionals/antivirals/antiviral-drug-resistance). NA inhibitors block the enzyme active site of NA, thereby, prevents the release and the spread of virions (McKimm-Breschkin, 2013). The Centres for Disease Control and Prevention (CDC) recommends the use of three influenza antivirals, which include oseltamivir (Tamiflu), zanamivir (Relenza) and peramivir (Rapivab) (www.cdc.gov/flu/professionals/antivirals/summary-clinicians). Another antiviral laninamivir (Inavir) has been approved for use in Japan since 2010 (www.daiichisankyo.com/media_investors/media_relations/press_releases/detail/005703). Zanamivir (Relenza) was the first neuraminidase inhibitor to be synthesized based on a transition state analog DANA (deoxydehydron-acetyl neuramic acid) and the crystal structure complex of neuraminidase and sialic acid. Relenza has a guanidino group substituted at the C4-OH of DANA and suffers from the limitation of not being orally administrable. To overcome this limitation, oseltamivir (Tamiflu) was designed (McKimm-Breschkin, 2013; Garman & Laver, 2005). Tamiflu is orally administered as a prodrug (oseltamivir phosphate), which is then converted to the active form (oseltamivir carboxylate) by the hepatic enzyme convertase. Peramivir and Laninamivir were subsequently developed (Figure 4) (McKimm-Breschkin, 2013; (www.daiichisankyo.com/media_investors/media_relations/press_releases/detail/005703). Recently Fu et al. synthesised a tetravalent zanamivir with inhibitory high activity against drug-resistant influenza viruses (Fu et al., 2016).
Studies have shown the residues that account for the catalytic function of NA are conserved among all the NA subtypes of influenza A and B viruses, and hence serve as the major targets for antivirals. The residues (N2 numbering) that constitute the active site and have direct contact with the substrates such as oseltamivir and zanamivir are Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371 and Tyr406 (Figure 5). The geometry of catalytic site is stabilized by the framework residues Glu119, Arg156, Trp178, Ser179, Asp/Asn 198, Ile222, Glu227, His274, Glu277, Asn 294, and Glu425 (Xu et al., 2008). Mutations in the catalytic site and framework residues can lead to drug resistance. According to a global update on the susceptibility of influenza virus to NA inhibitors during the 2013-2014 season, ~ 98% of the circulating viruses were found sensitive to all four influenza antivirals and only 2% showed resistance to at least one drug, mainly oseltamivir. A large cluster of NA inhibitor resistant influenza A(H1N1)pdm09 viruses, with the H275Y substitution, were detected in Japan and other countries such as China and the USA (Takashita et al., 2015). The antiviral surveillance reports from National Institute of Infectious Diseases, Japan, during 2013-2014 influenza seasons showed ~4.1% resistance to both oseltamivir and zanamivir (www.nih.go.jp/niid/en/influ-resist-e/5655-flu-dr-e20150518). Resistant viruses are likely to increase in future due to the increasing use of NA inhibitors in recent times. There is, therefore, an urgent need to develop newer drugs and vaccines that can impart long-lasting and broader range of protection against influenza (McKimm-Breschkin, 2013).
Figure 5: Active site of influenza neuraminidase in complex with oseltamivir
The active site is represented in surface view. Amino acid residues of catalytic site (PDB: 3TI6) that interact with the drug are shown in sticks representation and coloured cyan (carbon). Oseltamivir substrate is shown as ball and stick representation and coloured green (carbon). The H-bonds are represented in dotted lines and corresponding distances are labeled.

2.4 Neuraminidase antigenic domains
The crystal structure of four NA-specific antibodies have been elucidated to date (Malby et al., 1994; Tulip et al., 1992; Venkatramani et al., 2006; Wan et al., 2015). The first crystal structure of NA antigen-antibody complex was neuraminidase N9 and monoclonal antibody NC41 (Tulip et al., 1992). Antigenic epitopes of N9 were discontinuous and comprised 19 residues with large surface areas buried by the interaction. Subsequently, another antibody N10 was synthesized with almost 80% overlap of the binding site with NC41 (Malby et al., 1994). In 2006, the crystal structure of N2 in complex with Mem5 was determined at a resolution of 2.1Å and exhibited
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novel patterns of water-mediated H-bond interactions that stabilized the complex (Venkatramani et al., 2006). Residues of NA at 198, 199, 220, and 221 contributing to the antigenic domain were found to be mutated in the viruses isolated after 1998, affirming that Mem5 binds to an epidemiologically important antigenic site (Gulati et al., 2002; Venkatramani et al., 2006). Recently, Wan H et al. crystallized an antibody CD6 in complex with NA of influenza A(H1N1)pdm09 virus that reveals a unique antigenic epitope, the light-chain complementarity determining regions (LCDRs) binding to one NA monomer, the heavy-chain CDRs 1 and 2 binding to the other monomer, and the HCDR3 binding to both the monomers. HCR1 interacts with Pro93, Val94, Ser95, Trp358, Trp375, Ser388, Ile389, Asn449, Ser450, and Asp451. HCR2 interacts with Asn355, Pro377, and Asn378 of the same monomer. HCR3 interacts with Asn449, Ser450, Asp451 of one monomer and Ile216, Lys262, Ile263, Val264, Lys265, and Ser266 of the other NA monomer. LCR interacts with residues Trp219, Arg220, Gln250, Ala251, Ser252, Lys254, Ser266, Val267, Glu268, and Asn270 (PDB: 4QNP; Figure 6) (Wan et al., 2015). More crystal structures of NA antigen-antibody complex should be published, as the information will be useful in designing NA-based vaccines.

![Figure 6: NA protein of Influenza A(H1N1)pdm09 in complex with antibody CD6](image)

The NA-CD6 model based on PDB ID: 4QNP is shown. NA is shown in cartoon and coloured in green (chain A) and yellow (chain B). Antibody heavy and light chains are shown in surface and are respectively coloured in blue and magenta. The antibody binds to the lateral surface of two NA monomers (Wan et al., 2015). NA residues that bind to the antibody are shown as dotted spheres.
2.5 Evolution of neuraminidase gene

A study on the evolution of hemagglutinin and neuraminidase genes in the Province of Que'bec (Canada) during the three influenza seasons (1997–2000) reported that amino acid substitutions in the NA sequences occur at a slower rate (0.45-1%) compared to HA (1-2%) (Abed et al., 2002). Sandbulte et al. showed that the antigenic drift in NA is slower and dissimilar to the antigenic drift of HA among H1N1 and H3N2 seasonal influenza vaccine viruses (Sandbulte et al., 2011). A recent study on the entire genome analysis of influenza A(H3N2) viruses, circulating between 1968 and 2011, has documented that amino acid substitutions in HA1 subunit occurred at a higher rate of 14.9x10^{-3}/site/year compared to 9.1x10^{-3}/site/year substitution rate in NA (Westgeest et al., 2014). The antigenic analysis of crystallized antibody CD6 in complex with influenza A(H1N1)pdm09 virus NA reveals the epitope is mostly conserved even after several years of circulation with mutations of some residues occurring at lower rates (Wan et al., 2015). All these studies emphasize that the evolution of NA is slow paced, that renders the NA antibodies capable of offering a long-term protection.

2.6 Role of anti-neuraminidase antibodies

NA-specific antibodies may not be effective in preventing infection but do inhibit the spread of the virus, thereby, reducing the severity of the disease (Schulman et al., 1968). Epidemiological evidence suggests that the reduced impact of the 1968 Hong Kong flu (H3N2) virus could be attributed to the presence of pre-existing NA antibodies produced against the Asian Flu (H2N2) virus (Monto & Kendal, 1973). Past studies have demonstrated no difference in the immunogenicity of HA and NA glycoproteins. Therefore, if a balanced formulation or the supplementation of conventional trivalent vaccines with purified N1 and N2 is used, it will induce a more balanced response, avoiding the usual HA-skewed response that occurs towards the available vaccines (Johansson et al., 1989; Johansson et al., 2002). It was also postulated that the antigenic competition between HA and NA results in greater B- and T-cell priming to HA antigen, supposedly due to a higher number of HA proteins found on the virion surface and its amplified presentation to the antigen recognition cells of the immune system (Johansson et al., 1987). Purified preparations of vaccines containing influenza A virus N2 neuraminidase are
proven to be immunogenic and non-toxic in humans (Kilbourne et al., 1995). Couch et al. showed that the naturally occurring influenza NA inhibiting antibody is an independent predictor of immunity even in the presence of HA inhibiting antibody (Couch et al., 2013). A study has reported that monoclonal antibodies developed against a conserved epitope of NA (amino acid 222-230) inhibited all 9 subtypes (N1-N9), providing evidence for consideration of NA in universal influenza vaccine development (Doyle et al., 2013b).

The role of NA antibodies has been demonstrated by different vaccines such as affinity chromatography purified NA vaccine (Hocart et al., 1995), plasmid DNA encoding NA (Chen et al., 1999; Zhang et al., 2005), mammalian expression system (Bosch et al., 2010), baculovirus derived recombinant NA protein (Kilbourne et al., 2004; Brett & Johansson, 2005) and VLP based vaccines co-expressing NA with HA and/or M proteins (Bright et al., 2007; Kang et al., 2009b). Several approaches have been used to estimate the antibodies against NA such as the traditional thiobarbituric acid assay. However, enzyme-linked lectin assay (ELLA) is considered a better practical and accurate method as the results are subtype specific and reproducible (Couzens et al., 2014).

2.7 Current influenza vaccines

The CDC’s advisory committee on immunization practices (ACIP) recommends the use of live attenuated influenza vaccine (LAIV), inactivated influenza vaccine (IIV), and recombinant influenza vaccine (RIV) to prevent influenza (www.cdc.gov/mmwr/preview/mmwrhtml/rr6207a1.htm#RecombinantInfluenzaVaccine). Six new influenza vaccines have been approved since 2012, which include 1) Fluarix: quadrivalent IIV; 2) Flumist: quadrivalent LAIV; 3) Flulaval: quadrivalent IIV; 4) Fluzone: quadrivalent IIV; 5) Flucelvax: cell culture based trivalent IIV; 6) Flublok: trivalent recombinant HA vaccine (www.cdc.gov/mmwr/preview/mmwrhtml/rr6207a1.htm#Recombinant_InfluenzaVaccine). These current vaccines are standardized mainly on the basis of the HA content (Han & Marasco, 2011), with varying amounts of other proteins, such as NA. The immune response to vaccination is estimated by measuring antibodies against HA using hemagglutination inhibition (HAI) assay, as these antibodies against HA are
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best characterized for offering protection against the circulating strains of influenza (Bright et al., 2009). The WHO conduct meetings twice a year in February and September to recommend the vaccine strains for influenza season in the Northern and Southern hemispheres, respectively (www.who.int/influenza/vaccines/virus/recommendations/). During the 2014-2015 influenza season, the virus activity was moderately high especially in the United States (late 2014 and beginning of 2015) due to the circulation of an antigenically distinct influenza A(H3N2) virus. Vaccination offered reduced protection as the vaccine strain showed only 48% similarity with the drifted strain (www.emergency.cdc.gov/han/han00374.asp). The HAI assay of the drifted viruses with ferret antisera showed reduced inhibition due to amino acid changes in the HA protein, which led to the changes in the WHO vaccine strain recommendations for the 2015-2016 season (www.who.int/influenza/vaccines/virus/recommendations). Several observations pointed at standardizing the amounts of NA along with HA protein in influenza vaccine formulations in order to compensate for the variations due to HA, to reduce and better contain the severity of influenza epidemics.

Couch et al. conducted a study to evaluate the anti- HA and anti-NA responses to six trivalent inactivated vaccines (TIV). The findings revealed that all TIV with similar HA content induced similar HA antibodies in healthy adults, but all vaccines did not induce similar NA responses since the vaccines contained varying amounts of NA. NA antibodies induced by LAIV were lower than TIV (Couch et al., 2012). The stability of neuraminidase in an inactivated influenza vaccine showed that NA immunogenicity is consistent throughout the shelf life of the vaccines (Sultana et al., 2014). Due to the lack of appropriate analytical methods, the guidelines for the standardization of NA have not been developed (Getie-Kebitie et al., 2013). Recent studies have demonstrated that the mass spectrometric techniques such as LC-MS and LC/MS/MS can simultaneously quantify HA and NA antigens in various vaccine preparations (Creskey et al., 2012; Williams et al., 2012).

2.8 Neuraminidase protein in influenza vaccine

The NA protein has been a well-established and important target for the treatment of influenza,
but the approved NA vaccines are not a reality yet. All the above-mentioned studies suggest that NA is a potential target for future influenza vaccines, but this does not lead to the replacement of current vaccines with the NA vaccines. NA antibodies are infection permissive and only reduce the disease severity, whereas the HA antibodies prevent infection (Johansson et al., 1989). Thus, vaccines containing standardized amounts of both HA and NA glycoproteins are required to provide a complete protection against influenza. Studies have demonstrated that HA: NA ratios are different for different subtypes and also strains within a subtype (Getie-Kebtie et al., 2013). Hence, the strain specific NA content and the HA: NA ratio in such vaccines needs to be standardized. Since, there is no approved analytical method to standardize the NA content in vaccines (Williams et al., 2012) the best approach could be alternative vaccines, which will induce NA antibody production in sufficient amounts such as the VLP-based or recombinant vaccines.

2.9 Virus-like particle based influenza vaccines

Recently, studies have focused on developing non-replicating VLPs as the alternative for conventional influenza vaccines. VLPs can be produced from both non-enveloped and enveloped viruses (Kang et al., 2009a). VLP based vaccines stimulate T and B cells and induce better immune responses to both HA and NA antigens (Behzadian et al., 2013; Kang et al., 2009a; Smith et al., 2013). Such vaccines are immunologically similar to live vaccines, but are safer at the same time as there is no nasal shedding or reversion to virulent form (Song et al., 2010). The details of some VLP based influenza vaccines containing NA protein are summarized in Table 1. Some studies have shown that VLPs containing NA along with other proteins can induce heterologous protection. Wu et al. developed a H5M2eN1 VLP which induced high titers of NA antibodies and conferred homologous (H5N1 virus) and heterologous (H1N1 virus) immunity (Wu et al., 2012). Some studies have shown that seasonal influenza vaccines can induce partial immunity against highly pathogenic H5N1 (Ding et al., 2011). An NA-VLP expressing NA and M of H1N1 influenza virus conferred 100% immunity against infection by homologous H1N1 virus as well as heterologous H3N2 virus (Quan et al., 2012).
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#### Table 1: VLP based influenza vaccines containing NA protein

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Components</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3N2</td>
<td>H3+N2+M1</td>
<td>Mice and ferrets vaccinated with the VLPs based on HA concentration in dose sparing experiment had high titters of HA antibodies. Animals vaccinated with high doses of VLPs had anti-NA antibodies.</td>
<td>Bright et al., 2007</td>
</tr>
<tr>
<td>H5N1</td>
<td>H5+N1+M1</td>
<td>Mice vaccinated with H5N1 VLP from a clade 2 isolate showed cross-clade protective immune response.</td>
<td>Bright et al., 2008</td>
</tr>
<tr>
<td>H5N1</td>
<td>H5+N1+M1</td>
<td>Ferrets vaccinated with H5N1 clade 2.1 VLP were protected from homologous clade 2.1 strain as well as heterologous clade 1, 2.2, and 2.3 H5N1 strains.</td>
<td>Mahmood et al., 2008</td>
</tr>
<tr>
<td>H5N1</td>
<td>H5+N1+M1</td>
<td>Mice vaccinated with H5N1 clade 1 and 2 VLP individually or a mixture showed homologous as well as heterologous protection against clade 2.3 viruses. Mice vaccinated with clade 1 VLP were protected against both clade 1 and 2 virus, but mice vaccinated with clade 1 VLP were protected against only clade 1 virus.</td>
<td>Crevar &amp; Ross, 2008</td>
</tr>
<tr>
<td>H5N1</td>
<td>H5+N1+M1</td>
<td>Mice vaccinated intranasally with H5N1 VLP developed long-term protective immune response against homologous virus infection.</td>
<td>Kang et al., 2009b</td>
</tr>
<tr>
<td>H9N2</td>
<td>H9+N2+M1</td>
<td>The VLPs elicited antibodies and protected BALB/c mice by inhibiting replication of H9N2 virus.</td>
<td>Pushko et al., 2007</td>
</tr>
<tr>
<td>H9N2</td>
<td>H9+N2+M1</td>
<td>Immunization of BALB/c mice with H9N2 VLP containing novasome adjuvant improved the protection against H9N2 virus, but mice vaccinated with clade 1 VLP were protected against only clade 1 virus.</td>
<td>Pushko et al., 2007</td>
</tr>
<tr>
<td>H7N9</td>
<td>H7+N9+M1</td>
<td>H7N9H7N9 VLP vaccine with iSCOMATRIX adjuvant elicited high levels of anti-HA and anti-NA antibodies in mice.</td>
<td>Fries et al., 2013</td>
</tr>
</tbody>
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