Objective 2

6. Objective 2: Development of influenza virus-like particles expressing HA, NA (N1 & N2) and M protein and characterization of its immunogenicity.

This chapter details the methodology used for construction, expression and immunogenicity testing of VLPs containing HA, N1, N2, and M protein. The results of expression and inoculation of VLPs in animal models have also been discussed.

6.1 Materials and methods

6.1.1 Ethical Clearance

Institutional Animal Ethics Committee (IAEC), Manipal University, Manipal (Certificate IAEC/KMC/58/2013), approved the study (Annexure 2).

6.1.2 RT-PCR for amplification of M and HA gene of influenza A(H1N1)pdm09 virus

For the production of VLPs, PCR amplified full length genes of HA, NA (N1 & N2), and M proteins were required. The isolate A/Trivandrum/MCVR261/2009(H1N1) virus was used for amplification of N1, HA and M gene and the isolate A/Calicut/MCVR4536/2010(H3N2) virus was used for amplification of N2 gene. Primers and protocol used for amplifying the full length NA gene of influenza A(H1N1) and influenza A(H3N2) virus isolates is mentioned in section 5.1.5. The reagents and PCR cycling conditions used for the amplification of M and HA gene were same as N1 and N2 protocol, but primers specific for amplification of M and HA gene were used (Table 15). After amplification, the PCR products were analyzed using 0.8% agarose gel. The amplified product appeared as a single band ~1000bp for M gene (Figure 17a) and ~1700bp for HA gene (Figure 17b). The PCR products were purified using a PCR purification kit (Qiagen, Hilden, Germany). Following amplification the HA and M gene nucleotide sequences were determined by DNA sequencing.
Table 15: Primers for amplification of full length M and HA gene of influenza A(H1N1)pdm09 viruses

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>bp</th>
<th>Tm(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA1F</td>
<td>AGCAAAAGCAGGGGAAAATA</td>
<td>20</td>
<td>62.4</td>
</tr>
<tr>
<td>HA1R</td>
<td>AGTAGAAACAAGGGTGTTTT</td>
<td>20</td>
<td>54.4</td>
</tr>
<tr>
<td>M1F</td>
<td>AGCAAAAGCAGGTAGATATT</td>
<td>20</td>
<td>54.8</td>
</tr>
<tr>
<td>M1R</td>
<td>AGTAGAAACAAGGTAGTTTT</td>
<td>20</td>
<td>50.7</td>
</tr>
</tbody>
</table>

Figure 17: Gel picture of amplified PCR product
(a) M gene of size ~1000bp (b) HA gene of size ~1700bp.
6.1.3 RT-PCR for including restriction enzyme sites into N1, N2, HA, and M genes

The primers designed (Table 16) using SnapGene software (GSL Biotech LLC, Chicago, IL) were used to amplify and insert restriction enzyme sites Sall and HindIII into N1 and N2 genes, NotI and SphI into the HA gene, and EcoRI and Sall into M gene. The PCR products of the individually amplified genes (N1, N2, HA, and M) were used as templates. Four separate PCR reactions (one for each gene) were carried out using QIAGEN® OneStep RT-PCR kit (Qiagen, Hilden, Germany). Cycling conditions were as per the manufacturer's instructions with an annealing temperature of 50°C. After amplification, PCR products were analyzed using 0.8% agarose gel. A product size of ~1450bp (Figure 18a), ~1450bp (Figure 18b), ~1800bp (Figure 19a), and ~1000bp (Figure 19b) was obtained for N1, N2, HA, and M gene respectively, but some non-specific amplification products were observed during analysis. In order to remove the non-specific amplification products, gel extraction was performed using GenElute™ gel extraction kit (Sigma, MO, USA).

Table 16: Primers for inserting restriction enzyme sites and amplification of full length N1, N2, HA, and M genes

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Restriction enzyme site</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1enzF</td>
<td>gtcgacATGAATCCAAACCCAAAA</td>
<td>Sall</td>
</tr>
<tr>
<td>N1enzR</td>
<td>aagcttAGAAACAAGGGAGTTTTT</td>
<td>HindIII</td>
</tr>
<tr>
<td>N2enzF</td>
<td>gtcgacATGAATCCAAATCAAAAG</td>
<td>Sall</td>
</tr>
<tr>
<td>N2enzR</td>
<td>aagcttGAAACAAGGGAGTTTTCTA</td>
<td>HindIII</td>
</tr>
<tr>
<td>H1enzF</td>
<td>gcggccgc ATGAAGGCAATACTAG</td>
<td>NotI</td>
</tr>
<tr>
<td>H1enzR</td>
<td>gcatgcGAAACAAGGGGTGTTPTTT</td>
<td>SphI</td>
</tr>
<tr>
<td>M1enzF</td>
<td>gatccATGAGTCTTCTAACCGAGG</td>
<td>EcoRI</td>
</tr>
<tr>
<td>M1enzR</td>
<td>gtcgacATGAGAAACAAGGGTAGTTTTTACT</td>
<td>Sall</td>
</tr>
</tbody>
</table>
Figure 18: Gel picture of NA gene with restriction sites inserted and amplified by PCR
(a) N1 gene with SalI and HindIII restriction sites. (b) N2 gene with SalI and HindIII restriction sites.

Figure 19: Gel picture of HA and M genes with restriction sites inserted and amplified by PCR
(a) HA gene with NcoI and SphI restriction sites.
(b) M gene with EcoRI and SalI restriction sites inserted and amplified by PCR.
6.1.4 Cloning of N1, N2, HA and M genes containing restriction sites into TA vector

In order to make more copies and store cloning of N1, N2, HA, and M was performed following the methodology mentioned in section 5.1.6. Plasmid DNA was isolated following the protocol mentioned in section 5.1.7 and analyzed by agarose gel electrophoresis (0.8%). A product size ~3kb (N1 and N2), ~2.7kb (M), and ~3.5kb (HA) were considered successful transformation and further confirmed by DNA sequencing. Glycerol stocks were prepared for all the four plasmids.

6.1.5 Restriction digestion and cloning into pFastBac vector

For cloning of N1 gene into pFastBac vector (Invitrogen, CA, USA), both the pFastBac vector and the TA vector (containing N1 gene) were double digested with FastDigest restriction enzymes (Thermo Scientific, Vilnius, Lithuania) Sall and HindIII. Similarly, for cloning N2, HA, and M gene both the vectors were double digested with FastDigest restriction enzymes Sall and HindIII, NotI and SphI, and EcoR1 and Sall, respectively. The digested products were analysed using 0.5% gel using GreenView™ gel loading dye (Chromous Biotech, KA, India). The DNA fragments of interest were extracted from slices of agarose gel using GenElute™ Gel Extraction Kit (Sigma, MO, USA). For ligation, the insert to vector ratio used was 3:1. The transformation was performed using One shot® MAX efficiency® DH5α competent cells (Invitrogen, CA, USA). After transformation, the cells were plated onto LB media containing ampicillin. The plates were incubated at 37°C overnight and checked for large-sized white colonies. Plasmid DNA was isolated following the protocol already mentioned in section 5.1.7. Confirmation for successful transformation and incorporation of the insert was carried out by double digesting the recombinant plasmid DNA with specific restriction enzyme. Digested products were analyzed using agarose gel electrophoresis (0.8%). The results of gel analysis showed two bands on gel, plasmid (~4500bp) and NA insert (~1450bp; Figure 20a) or HA insert (~1800bp; Figure 20b) or M insert (~1000bp; Figure 21)
Figure 20: Gel picture of recombinant pFastBac vector
(a) pFastBac containing NA genes double digested with restriction enzymes. Lane 1 shows N1 gene (~1450bp) + pFastBac (~4500bp). Lane 2 shows N2 gene (~1450bp) + pFastBac (~4500bp). (b) pFastBac containing HA gene double digested with restriction enzymes. Lane 2 shows HA gene (~1800bp) + pFastBac (~4500bp).

Figure 21: Gel picture of recombinant pFastBac vector containing M gene double digested with restriction enzymes. Lane 1 and 2 show M gene (~1000bp) + pFastBac (~4500bp).
6.1.6 Ligation of N1 and N2 gene fragments by overlap extension (OE) PCR

Two sets of primers were designed in SnapGene software (GSL Biotech LLC, Chicago, IL) for OE PCR (Table 17). The first set of primers was used to amplify N1 gene along with the promoter region of the pFastBac vector (Figure 22). Phusion High-Fidelity PCR kit (Thermo Scientific, Vilnius, Lithuania) was used for amplification. The cycling conditions were according to the manufacturer's protocol and the product size was \(~1670\text{bp}\) (Figure 23a).

The second set of primers was used to amplify N2 gene along promoter region of pFastBac vector (Figure 22). The forward primer of the second set was designed such that it has the complimentary bases to reverse primer of first set (Table 17). The Phusion High-Fidelity PCR kit (Thermo Scientific, Vilnius, Lithuania) was used and the product size was \(~1900\text{bp}\) (Fig 23a).

In the next step, in order to obtain a single ligated fragment that contains both N1 and N2 genes, the products from step 1 and 2 were mixed and a PCR was setup for 25 cycles with all the master mix components except primers. Due to complimentary overlapping regions between the reverse region of template 1 and the forward region of template 2 both the strands annealed and a single fragment of size \(~3500\text{bp}\) was obtained (Figure 23b), but some non-specific amplification products were also observed during analysis. In order to remove non-specific amplification products, gel extraction was performed using GenElute™ gel extraction kit (Sigma, MO, USA). DNA sequencing was performed using primers N1proF and N2proR to confirm that OE PCR ligated N1 and N2 genes successfully. Sequence analysis showed both N1 and N2 sequences in a single segment.
Table 17: Primers for inserting restriction complimentary bases and ligating N1 and N2 gene fragments

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1proF</td>
<td>ATAGTTCTAGTGTTGGCTACGTA</td>
</tr>
<tr>
<td>N1proR</td>
<td>TCTAGTACTTCTCGACAAAGCTT</td>
</tr>
<tr>
<td>N2proF</td>
<td>AAGCTTGTCCAGAATCTAGATACTCCGGAATATTAATGATC</td>
</tr>
<tr>
<td>N2proR</td>
<td>GGATCTCCTGGCTCAAGCAGTGA</td>
</tr>
</tbody>
</table>

Note: Complimentary overlapping bases are shown in bold and underlined

Figure 22: Snapshot of the steps for inserting restriction complimentary bases and ligating N1 and N2 gene fragments.
Figure 23: Gel picture of OE PCR products

(a) NA gene amplified along with promoter region of the pFastBac vector. Lane 1 shows N1 gene (~1670bp) and Lane 2 shows N2 gene (~1900bp). (b) Ligated N1+N2 product. lane 1, 2 & 3 shows ligated N1+N2 product (~3500bp).

6.1.7 Construction of cassette containing HA, NA (N1 & N2), and M

6.1.7a Construction of pFastBacN1N2

N1N2 gene segment and pFastBac vector (Invitrogen, CA, USA) were double digested with FastDigest restriction enzymes (Thermo Scientific, Vilnius, Lithuania) SnaB1 and AvrII. The digested products were analyzed using 0.5% gel. The DNA fragments of interest were extracted from slices of agarose gel and transformation was performed as mentioned in section 6.1.4. The isolated plasmid DNA was analyzed by agarose gel electrophoresis (0.5%). A product size ~8000bp was considered successful transformation (Figure 24). Glycerol stocks of DH5α containing pFastBacN1N2 were prepared.
6.1.7b Construction of pFastBacMN1N2

Plasmid pFastBacN1N2 was double digested with FastDigest restriction enzymes SnaB1 and AvrII. Similarly, plasmid pFastBacM was double digested with restriction enzymes HpaI and AvrII (Figure 25). The digested products were loaded onto 0.5% agarose gel. The DNA fragments of interest, N1N2 gene (~3500bp) and pFastBacM (~6000bp), were extracted and transformation in DH5α was performed as mentioned in section 6.1.4. The isolated DNA was analyzed by agarose gel electrophoresis (0.5%) and a product size ~9000bp was considered successful transformation. Glycerol stocks of DH5α containing plasmid pFastBacN1N2 were prepared. For confirmation, the plasmid DNA was double digested with SnaBI and AvrII, and analysed by 0.5% agarose gel electrophoresis. Results of gel analysis showed two bands of size ~4900bp and ~4200bp (Figure 26).
Figure 25: Snapshot of the steps for constructing pFastBacMN1N2

Figure 26: Agarose gel picture of pFastBacN1N2M
double digested with restriction enzymes SnaBI and AvrII. Lane 1 shows products
~4200bp and ~4900bp.
Objective 2

6.1.7c Construction of pFastBacMN1N2HA

The plasmid pFastBacN1N2M was double digested with restriction enzymes Eco53KI and SphI. Plasmid pFastBacHA was double digested with restriction enzymes SnaBI and SphI (Figure 27). The digested products were loaded onto 0.5% agarose gel. DNA fragments of interest, pFastBacN1N2M (~9000bp), and HA (~1900bp) were extracted from slices of agarose gel extracted and transformation in DH5α was performed as mentioned in section 6.1.5. The isolated DNA was analyzed by agarose gel and a product size ~11000bp was considered successful transformation. Glycerol stocks of DH5α containing plasmid pFastBacN1N2MHA were prepared. For confirmation, plasmid DNA was double digested with SnaBI and SphI, and analysed by 0.5% agarose gel electrophoresis. The results of gel analysis showed two bands of size ~8000bp and ~3000bp (Figure 28).

Figure 27: Snapshot of the steps for constructing pFastBacMN1N2HA
Figure 28: Gel picture of pFastBacN1N2MHA containing double digested with restriction enzymes SnaBI and SphI. Lane 1 shows products ~8000bp and ~3000bp.

6.1.8 Transformation of pFastBacN1N2MHA donor plasmid into DH10Bac™

The transformation was carried using DH10Bac™ (Invitrogen, CA, USA) competent cells following protocol mentioned in Bac-to-Bac® Baculovirus expression system manual (Invitrogen, CA, USA). After transformation, the cells were plated on LB media containing gentamicin, kanamycin, tetracycline, bluo-gal, and IPTG. Plates were incubated at 37°C for 48 hrs. The white colonies were subcultured onto fresh plates and after confirmation of white phenotype, a single white colony was inoculated into LB media containing kanamycin, gentamicin, and tetracycline. After overnight incubation, the bacmid DNA was extracted using plasmid plus midi kit (Qiagen, Hilden, Germany) following kit protocol. The isolated plasmid DNA was analyzed by agarose gel electrophoresis (0.5%). Two bands of size ~23000bp and >9000bp were observed (Figure 29). The bacmid DNA fragments of interest (~23000bp) were extracted from the slices of agarose gel using GenElute™ Gel Extraction Kit (Sigma, MO, USA).
Objective 2

Figure 29: Gel picture of isolated bacmid DNA
Lane 1 shows two products of size ~23000bp and >9000bp.

6.1.9 Transfection of Spodoptera frugiperda (Sf9) cells to generate recombinant baculovirus

The recombinant bacmid DNA was transfected into actively growing Sf9 cells (Gibco, NY, USA) with viability of >97%. 9x10^5 cells in 2ml of Sf900 II serum free media (Invitrogen, CA, USA) seeded into 6-well plate were used. Transfection was performed using CellFectin reagent (Invitrogen, CA, USA) following the protocol mentioned in Bac-to-Bac<sup>®</sup>Baculovirus expression system manual (Invitrogen, CA, USA). After transfection, the plates were incubated at 27°C and monitored for development of cytopathic effect (CPE) once every 24hrs for seven days. The culture supernatant was clarified by centrifugation for 5mins at 500xg and passaged once in Sf9 cells to produce P1 working stock for VLP bulk production.

6.1.10 VLP production and concentration

Twenty-five 6-well plates with Sf9 cells at a cell density of 2x10^6 were inoculated with P1 stock and incubated at 27°C. The plates were monitored for CPE once every 24hrs and VLPs from culture supernatant were harvested 72hrs post-infection. Culture supernatants containing VLPs were
clarified by using low-speed centrifugation at 2000xg for 20 min at 4°C. The VLPs were pelleted by ultracentrifugation at 100,000xg for 60 mins at 4°C using Sorvall swinging bucket rotor (Thermo scientific, Ohio, USA). The pellets were resuspended in 1ml of phosphate buffered saline (PBS) at 4°C overnight.

### 6.1.11 VLP purification

Resuspended VLPs were purified through 20%-60% discontinuous sucrose gradient ultracentrifugation. The densest sucrose solution (60%) was layered at the bottom and successive lower density sucrose solutions (50%, 40%, 30%, and 20%) were layered on top. Resuspended VLPs were layered on the 20% sucrose solution and ultracentrifugation was performed at 27000rpm for 2hrs at 4°C. Fractions were collected from the sucrose layers using 1ml syringe and analyzed using SDS-PAGE, western blot, HA, and NA assay. The fractions containing VLP were pooled.

### 6.1.12 Detection and quantification of VLPs

#### 6.1.12a Electron microscopy

Purified VLP samples were adsorbed onto carbon coated grids and stained with 2% phosphotungstic acid for 5-10 min. Excess stain was removed and air dried for 1-3 minutes. Influenza VLPs were then visualized by using transmission electron microscope (Philips, Columbus, OH) at magnifications ranging from 60,000× to 100,000× at the National institute of Virology, Pune.

#### 6.1.12b Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

SDS-PAGE (4-10%) and Coomassie brilliant blue staining (Sigma, MO, USA) were used to verify the VLP protein expression. The proteins separated on SDS-PAGE were transferred onto a polyvinylidene (PVDF) membrane with the Mini-PROTEAN tetra cell (Bio–Rad, CA, USA). Following transfer, the membrane was blocked by 5% skimmed milk solution and then incubated overnight at 4°C with rabbit polyclonal antibodies (1:500 v/v) of H1 and influenza A(H1N1)
virus positive pooled human serum antibody for NA and M. Subsequently, the membrane was incubated with secondary anti-rabbit IgG/anti-human IgG horseradish peroxidase (HRP) conjugated antibodies (1:5000 v/v, Chemicon, EMD Millipore, MA, USA) and visualized using G box chemi XRQ (Syngene, Synoptics Ltd, Cambridge, UK). The expression of N2 protein could not be confirmed due to the lack of specific antibody.

6.1.12c Estimation of total protein

Total protein was estimated by bicinchoninic acid (BCA) method using Pierce™ BCA Protein Assay (Thermo Scientific, IL, USA). The total protein was estimated following manufacturer's guidelines and absorbance was measured at 562nm. The total protein was determined in reference to standard concentration of a known protein such as bovine serum albumin (BSA).

6.1.12d Hemagglutination and neuraminidase assay

A series of twofold dilutions of influenza VLPs in PBS (25μl) were prepared and incubated at room temperature for 1hr with an equal volume of 0.75% human red blood cells. Hemagglutination unit (HAU) was determined as the highest dilution of VLP showing visible agglutination of red blood cells.

Neuraminidase activity of VLPs assessed using substrate 20-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA; Sigma, MO, USA) as mentioned in section 5.1.11.

6.1.13 Immunogenicity testing

In order to determine the immunogenicity of VLPs, an exploratory study was conducted with seven female inbred BALB/c mice (6-8 weeks). Mice were inoculated intramuscularly on the left hind limb with ~1μg of VLPs in 50μl of PBS on day 0 and 21. The negative control group (7 mice) received PBS in place of VLPs. The mice were observed daily to monitor changes in body weight and record mortality. Blood samples (1% of mice body weight) were collected by retro-orbital plexus puncture before the primary inoculations and booster inoculations on days 0, 7, 21, 35 and 56.
In order to determine the concentration of VLP that induces the production of significant amount of antibodies, higher concentrations of VLPs (\(\sim 5\mu g\), \(\sim 10\mu g\), \(\sim 15\mu g\)) were also inoculated in three additional groups of mice (7 per group) and blood samples were collected.

### 6.1.14 Antibody responses to influenza VLPs

Antibody responses to influenza VLPs were assessed by HAI antibody assay and NAI antibody assay. HAI assay was performed by treating serum samples with receptor destroying enzyme (RDE) and serially diluted twofold in microtitre plates. An equal volume of (4 HAU/25\(\mu l\)) influenza A/California/07/2009(H1N1) virus antigen was added and incubated at room temperature for 1 hr. After the incubation, 0.5% of chick RBCs was added and incubated at room temperature for 30mins. The reciprocal of the highest dilution which contained non-agglutinated RBC was considered as HAI antibody titer.

Enzyme-linked lectin assay to measure NAI antibody titres was performed according to the protocol standardised by Couzens et al. Twofold dilutions of serum samples (heat treated) were added to the fetuin-coated plate. An equal volume of virus (A/California/07/2009(H1N1) or A/Washington/01/2007(H3N2)) was then added and incubated at 37\(^\circ\)C for 16-18hrs. After incubation, peanut agglutinin (PNA) conjugated to horseradish peroxidase (HRPO; Sigma, MO, USA) was added and plates were incubated at room temperature for 2hrs. o-phenylenediamine dihydrochloride (OPD) was then added as a substrate to all wells and incubated in dark for 10mins. The reaction was stopped by 1 N H\(_2\)SO\(_4\), and plates were read at 490nm (Couzens et al., 2014). The reciprocal of the highest dilution which resulted in at least 50% inhibition of maximum signal was considered as NAI antibody titre.

### 6.2 Results

#### 6.2.1 Analysis of recombinant bacmid

PCR was performed using pUC/M13 amplification primers in order to confirm that the N1N2MHA DNA was transposed into the bacmid. The primers were directed at sequences on either side of the miniattTn7site within the \(lacZ\alpha\)-complementation region of the bacmid.
Phusion High-Fidelity DNA polymerase (Thermo Scientific, Vilnius, Lithuania) was used and the product size of $\sim10000\text{bp}$ (Figure 30) was obtained indicating successful transposition of DNA into bacmid.

![Figure 30: Gel picture of PCR amplified bacmid DNA](image)

Lane 1 shows product of size $\sim10000\text{bp}$.

6.2.2 Transfection of Sf9 cells

The recombinant bacmid DNA was transfected into actively growing Sf9 cells (Gibco, NY, USA). On day 7, the transfected cells displayed CPE. In comparison with the negative control (no recombinant DNA) flask, the positive flask had enlarged cells with poor adherence the substrate and low cell density indicating successful transfection (Figure 31).
Figure 31: Cytopathic effect of recombinant baculovirus
(a) Negative control flask with increased number of cells.
(b) Flask showing signs of virus growth with enlarged round cells on day 7.

6.2.3 Electron microscopy

VLPs imaged by transmission electron microscopy resembled in size (~100 nm) and structure to influenza VLPs. VLPs were spherical, enveloped with protein spikes presumed to be HA and NA surface glycoprotein projecting from the surface of each particle (Figure 32).

Figure 32 a & b: Electron micrograph of negatively stained influenza VLPs
Bar indicates size in nm
6.2.4 Determination of recombinant proteins

To analyze the co-expression of the four recombinant proteins, the VLPs from Sf9 cell culture supernatants were harvested after 72 hours. VLPs were pelleted by ultracentrifugation and resuspended in PBS. The resuspended VLPs were purified through 20%-60% discontinuous sucrose gradient ultracentrifugation. Majority of the purified VLPs were found to be layered in 50-60% sucrose fraction (Figure 33), but all the layered were collected and the total protein was electrophoresed on 4 - 10% polyacrylamide gel SDS-PAGE and stained with coomassie. Two protein bands of ~70 and ~27 kDa, representing the HA and M respectively, were detected as clear bands after staining (Figure 34), but the protein band of ~55kDa of N1/N2 was detected as a very faint band. The western blot analysis of separated proteins on SDS-PAGE, probed with anti-HA, anti-NA, and anti-M1 antibodies proved the specificity of the detected bands, and demonstrated simultaneous and independent expression of the all the recombinant influenza virus proteins.

![Figure 33: Sucrose gradient ultracentrifugation](image)

VLPs purified by layering onto 20-60% fractions. Purified VLPs were detected at 50-60% sucrose.
Figure 34: Determination of recombinant proteins

(a) SDS-PAGE and coomassie brilliant blue stained gel.
(b) Western blot analysis of VLP probed with anti-HA antibodies
(c) Western blot analysis of VLP probed with pooled influenza A(H1N1) virus positive human serum antibodies.

6.2.5 Quantification of VLP

The total protein was estimated to be $\sim 1 \text{mg/ml}$ by BCA method. The HA assay was performed to demonstrate the agglutination activity of sucrose cushion purified VLPs. HA activity was determined as $256 \text{HAU}/25 \mu l$ and the highest dilution showing agglutination was $\sim 1:256$ Neuraminidase activity of VLP estimated using MUNANA as substrate was $320 \text{RFU}$ and the highest dilution with NA activity was $\sim 1:4096$.

6.2.6 Antibody responses to VLPs

Four groups of BALB/c mice (6-8 weeks) were inoculated with intramuscularly with different concentrations of VLP ($\sim 1 \mu g$, $\sim 5 \mu g$, $\sim 10 \mu g$, and $\sim 15 \mu g$) on day 0 (prime) and day 21 (booster). Serum samples were collected at week 1, 3, 5 and 8 to measure antibody responses to vaccination.
At week 3, ~57% of mice vaccinated with ~1μg and ~5μg VLP and ~100% of mice vaccinated with ~10μg and ~15μg of VLP had measurable titers of NA and/or HA antibodies. At week 5, ~100% of mice vaccinated with ~1μg, 5μg, ~10μg, and ~15μg of VLP had measurable NA antibody titers. Anti-NA responses were evaluated using influenza A/California/07/2009(H1N1) virus and A/Washington/01/2007(H3N2) virus (Table 18 and 19). Day 35 sera from mice inoculated with ~1μg, 5μg, 10μg and 15μg showed anti-NA antibody titers of 140, 417, 560 and 383, respectively, against influenza A/California/07/2009(H1N1) virus. A twofold to fourfold rise in anti-NA antibody titres were observed in mice belonging to all the four group by week 5. Similar anti-NA responses were also demonstrated against influenza A/Washington/01/2007(H3N2) virus (Table 19). However, anti-HA antibody titers were low (10) compared to anti-NA antibody titers for all the four groups of mice in week 3. An increase in anti-HA antibody titer was observed by week 5, but by week 8 the anti-HA antibodies decreased by twofold (Table 20).

Table 18: Neuraminidase inhibition antibody assay using A/California/07/2009(H1N1)

<table>
<thead>
<tr>
<th>VLP (Dose)</th>
<th>Week 1</th>
<th>Week 3 (responders)</th>
<th>Week 5 (responders)</th>
<th>Week 8 (responders)</th>
</tr>
</thead>
<tbody>
<tr>
<td>~1μg</td>
<td>0</td>
<td>80 (3/7)</td>
<td>140 (7/7)</td>
<td>397 (7/7)</td>
</tr>
<tr>
<td>~5μg</td>
<td>0</td>
<td>97 (3/7)</td>
<td>417 (7/7)</td>
<td>577 (7/7)</td>
</tr>
<tr>
<td>~10μg</td>
<td>0</td>
<td>113 (4/7)</td>
<td>560 (7/7)</td>
<td>720 (6/6)</td>
</tr>
<tr>
<td>~15μg</td>
<td>0</td>
<td>129 (4/7)</td>
<td>383 (7/7)</td>
<td>689 (7/7)</td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>0 (0/7)</td>
<td>0 (0/7)</td>
<td>0 (0/7)</td>
</tr>
</tbody>
</table>
### Table 19: Neuraminidase inhibition antibody assay

using A/Washington/01/2007(H3N2)

<table>
<thead>
<tr>
<th>VLP (Dose)</th>
<th>Week 1</th>
<th>Week 3 (responders)</th>
<th>Week 5 (responders)</th>
<th>Week 8 (responders)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1 µg</td>
<td>0</td>
<td>50 (4/7)</td>
<td>88 (7/7)</td>
<td>320 (6/6)</td>
</tr>
<tr>
<td>-5 µg</td>
<td>0</td>
<td>115 (4/7)</td>
<td>400 (7/7)</td>
<td>674 (7/7)</td>
</tr>
<tr>
<td>-10 µg</td>
<td>0</td>
<td>137 (7/7)</td>
<td>853 (6/6)</td>
<td>960 (6/6)</td>
</tr>
<tr>
<td>-15 µg</td>
<td>0</td>
<td>162 (7/7)</td>
<td>720 (7/7)</td>
<td>869 (7/7)</td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>0 (0/7)</td>
<td>0 (0/7)</td>
<td>0 (0/7)</td>
</tr>
</tbody>
</table>

### Table 20: Hemagglutination inhibition antibody titre

<table>
<thead>
<tr>
<th>VLP (Dose)</th>
<th>Week 1</th>
<th>Week 3 (responders)</th>
<th>Week 5 (responders)</th>
<th>Week 8 (responders)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1 µg</td>
<td>0</td>
<td>10 (1/7)</td>
<td>26 (3/7)</td>
<td>32 (5/7)</td>
</tr>
<tr>
<td>-5 µg</td>
<td>0</td>
<td>10 (1/5)</td>
<td>80 (2/7)</td>
<td>30 (5/7)</td>
</tr>
<tr>
<td>-10 µg</td>
<td>0</td>
<td>10 (2/6)</td>
<td>60 (2/7)</td>
<td>40 (2/7)</td>
</tr>
<tr>
<td>-15 µg</td>
<td>0</td>
<td>10 (1/4)</td>
<td>120 (3/7)</td>
<td>87 (3/7)</td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>0</td>
<td>11 (4/7)</td>
<td>13 (7/7)</td>
</tr>
</tbody>
</table>

### 6.3 Discussion

Egg-based production of influenza vaccines have been the standard method since the 1940s and these vaccines elicit antibodies primarily against the HA protein. However, in recent years with the increasing demand for vaccines, faster and safer high-yielding production methods need to be established. Insect cell-derived influenza vaccines are considered a new alternative to the conventional egg-based vaccines. The insect expression system offers several advantages in
comparison to egg-based vaccines such as easy to handle, high yield, reduced manufacturing time, safe, and cost-effective (Krammer & Grabherr, 2010). The baculovirus expression vector systems (BEVS) have been used for almost three decades for the production of recombinant proteins in insect cells (van Oers et al., 2015). The BEVS have also been used for influenza virus VLP production in a number of studies and have shown promising results. The VLP-based influenza vaccines are proven to induce significant amounts of anti-HA and anti-NA antibodies in immunized mice (Behzadian et al., 2013; Bright et al., 2007; Pushko et al., 2005; Smith et al., 2013) unlike the currently licensed vaccines that primarily elicit anti-HA antibodies (Bright et al., 2007).

In the present study, we have designed and constructed a recombinant baculovirus to infect Sf9 cells and express VLPs composed of four proteins of influenza virus viz., H1, N1, and M from influenza A(H1N1) virus and N2 protein from influenza A(H3N2) virus. HA is the major surface protein of influenza virus and anti-HA antibodies are highly potent in inhibiting virus replication (Johansson et al., 1989), hence, the HA protein was incorporated in the VLP cassette. Our study on the analysis of NA gene sequences of influenza A(H1N1) and (H3N2) virus strengthens the fact that genetic variations in these genes occur at a slower rate and therefore the anti-NA antibodies can afford long-term protection. Further, to increase the spectrum of protection afforded by the influenza VLP both N1 and N2 proteins were incorporated since a strong N1 and N2 based immunity might be beneficial during the emergence of newer pandemic viruses with heterologous NA such as H2N2 or H5N1 (Wohlbold et al., 2015). Studies have documented that the M protein is the key element essential for the generation of influenza VLP and hence M protein was included.

For co-expression of all the four proteins, the individual gene segments were placed under the transcription control of polyhedron promoter. SV40 poly (A) signal element was used for the termination of transcription and improving translational efficiency. The recombinant baculovirus DNA constructed was then used for transfection of Sf9 cells and production of VLPs. Thompson et al. assessed the expression of influenza VLP in Sf9 and HEK293 expression systems and reported that VLP production in Sf9 cells was 35 times more than HEK293 cells, HA activity
was higher and VLPs had a more homogenous morphology (Thompson et al., 2015). VLPs produced using the insect cell culture system was purified by sucrose gradient ultracentrifugation. Purified VLPs were analysed and confirmed the expression of H1, N1 and M protein using electron microscopy, SDS-PAGE and western blot. The expression of N2 protein could not be confirmed due to the unavailability of N2 specific purified antibody. Currently, there are no total particle quantification methods for the influenza VLPs other than antigen based quantification such HA assay, NA assay, and single radial immunodiffusion (SRID) (Thompson et al., 2013). In our study, the total VLP protein estimated by BCA method was 1mg/ml, the HA activity was ~256HAU/25μl and the highest dilution of VLPs with NA activity was ~ 1:4096. SRID could not be performed due to unavailability of homologous reference HA antigens.

The novel VLP developed in the study was also tested for its immunogenicity by inoculating varying concentrations (~1μg, ~5μg, ~10μg and ~15μg) in mice model. The VLPs induced a significant amount of anti-NA and anti-HA responses in mice. Increased anti-NA responses may be attributed to the presence of two NA proteins (N1 and N2) in VLP. Experiments such as microneutralisation assay or plaque reduction neutralisation assay will be carried out in future to demonstrate homologous and heterologous immunity induced by the VLPs synthesized in this study.