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Induction of immunogenic response in Balb/c mice by virus-like particles designed using the influenza neuraminidase, hemagglutinin and matrix proteins

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Summary. – Influenza is a vaccine-preventable disease, however, vaccine production confronts the greatest challenge due to the annual emergence of mutated or newer subtypes of the influenza virus. Virus-like particles (VLPs) are the next generation vaccines trending the research domain. Several research groups have highlighted the usefulness of neuraminidase (NA) as a vaccine antigen. VLPs incorporating consistent amounts of NA are designed and tested for immunogenic potential. In this study, influenza VLPs containing hemagglutinin (HA), NA (N1 and N2), and matrix (M) proteins were designed and tested for immunogenicity in Balb/c mice. Two different concentrations of VLPs (5 µg or 10 µg) with and without 15 µg Quil-A adjuvant were injected to mice. VLPs at the 10 µg concentration administered in combination with 15 µg Quil-A adjuvant were found to induce good anti-NA and anti-HA antibody responses. The induction of immune response in mice supports the hypothesis that VLP-based vaccines are promising candidates for future vaccines.

Keywords: influenza; VLP; Quil-A adjuvant; immunogenicity; mouse model

Introduction

Influenza is an acute respiratory illness caused by the influenza virus. Influenza affects mainly the upper and/or lower respiratory tract, resulting in moderate to severe illness that can lead to hospitalization. The airborne nature of the influenza viruses facilitates widespread circulation, often culminating in seasonal epidemics in the temperate regions and at times in all-year-round epidemics in some tropical regions (WHO, influenza vaccines). Vaccination is one of the most effective public health measures with tremendous prophylactic potential, especially during influenza epidemics, nevertheless, the dissemination of antivirals becomes indispensable at the outset of an outbreak. Towards influenza prophylaxis, the CDC advisory committee recommends immunization practices that mostly include the use of licensed quadrivalent live attenuated influenza vaccine, inactivated influenza vaccine, or quadrivalent recombinant influenza vaccine. These vaccines are mainly standardized with respect to the hemagglutinin (HA) content (Jagadesh *et al.,* 2016a; Summary of Recommendations, CDC). Despite the wide availability of vaccines, influenza remains a pervasive public health problem due to the several limitations that exist both in terms of availability and effectiveness (Soema *et al.,* 2015).

The neuraminidase (NA) protein has been an important target for anti-influenza drug development, however, NA-based vaccines are not yet available on the market despite a decade-long research (Jagadesh *et al.,* 2016a). Comparatively, antibodies against NA protein, though reducing the disease severity, are more infection permissive

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Abbreviations: HA = hemagglutinin; H1 = HA 1; HAI = HA inhibition; M = matrix; NA = neuraminidase; N1 = NA 1; N2 = NA 2; NAI = NA inhibition; OE = overlap extension; VLP(s) = virus-like particle(s)

than the anti-HA antibodies, which resist infection better (Johansson *et al.,* 1989). Studies have shown that the NA gene encounters antigenic variation at a slower rate than the other surface glycoprotein HA. Thus, anti-NA antibodies have the potential to induce long-term protective immunity. In short, vaccines designed using standardized amounts of both HA and NA proteins might be required to provide complete and long-term protection against influenza (Jagadesh *et al.,* 2016a).

Recently, non-replicating VLPs, produced from both non-enveloped and enveloped viruses, are being considered as alternatives to conventional vaccines (Kang *et al.,* 2009). VLP-based vaccines are reported to induce an immune response similar to live vaccines and are safer at the same time (Song *et al.,* 2010). Influenza vaccines so far have been mostly developed based on the immunogenic response to HA protein, and not much has been tried using NA protein, considering the unavailability of standard techniques, solely aimed towards standardization of NA (Getie-Kebtie *et al.,* 2013). However, new-generation VLP-based vaccines, incorporating consistent amounts of NA, are eventually drawing the interest and attention (Eichelberger and Wan, 2014). Although a variety of influenza VLPs have been developed to date, VLP containing N1 and N2 proteins along with HA and M is a novel concept. The present study was therefore aimed at studying the immunogenicity of a novel VLP that expresses one HA protein (Hemagglutinin 1 (H1) protein, two NA proteins (Neuraminidase 1 (N1) and Neuraminidase 2 (N2)), and matrix (M) protein. The invention and the process have been filed for an Indian patent (Application No. 201741038086).

Materials and Methods

Amplification of HA, NA (N1 and N2) and M genes. A/Trivandrum/MCVR261/2009(H1N1) virus isolate was used for the amplification of N1, HA and M genes and A/Calicut/MCVR4536/2010(H3N2) virus isolate was used for the amplification of the N2 gene. Primers from Chan *et al's* study were used for amplifying the full-length H1, N1 and M genes of influenza A(H1N1) and Tang *et al's* primers were used for amplification of the N2 gene of influenza A(H3N2) virus (Chan *et al.,* 2006; Tang *et al.,* 2008). QIAGEN ®OneStep RT-PCR kit (Qiagen, Hilden, Germany) was used for cDNA synthesis and PCR amplification. Following amplification, the HA, N1, N2, and M gene nucleotide sequences were determined by DNA sequencing (Genbank ID MF629794.1, KM985427.1, KX697016.1, and MF629795.1, respectively).

Generation of recombinant bacmid DNA. Restriction enzyme sites *Sal*I-*Hind*III, *Not*I-*Sph*I, and *Eco*R1-*Sal*I were inserted into NA (N1 and N2), HA, and M gene, respectively. The N1 and N2 genes containing restriction sites were cloned into *Sal*I-*Hind*III digested pFastBac transfer vector (Thermo Fisher Scientific, MA, USA). Similarly, HA gene was cloned into *Not*I-*Sph*I digested pFastBac vector, and M gene into *Eco*R1-*Sal*I digested pFastBac vector. The resulting plasmids were designated as pFastBacN1, pFastBacN2, pFastBacHA, and pFastBacM, respectively. In order to obtain a single ligated fragment that contains both N1 and N2 gene, overlap extension (OE) PCR was performed using Phusion High-Fidelity PCR kit (Thermo Fisher Scientific, MA, USA). Two sets of primers were designed in SnapGene software (GSL Biotech LLC, Chicago, IL) for OE PCR. The first set of primers was used to amplify the N1 gene along with the promoter region of the pFastBac vector. The second set of primers was used to amplify the N2 gene along the promoter region of the pFastBac vector. The forward primer of the second set was designed such that it has the complementary bases to reverse primer of the first set. DNA sequencing was performed to confirm that OE PCR ligated N1 and N2 genes successfully. Sequence analysis showed both N1 and N2 sequences in a single segment (N1N2).

The N1N2 gene fragment was digested and cloned into *Sna*B1-*Avr*II digested pFastBac vector resulting in the pFastBacN1N2 vector. Plasmid pFastBacN1N2 was double digested with FastDigest restriction enzymes (Thermo Fisher Scientific, MA, USA) *Sna*B1-*Avr*II and ligated into *Hpa*I-*Avr*II digested pFastBacM resulting in plasmid pFastBacMN1N2. pFastBacMN1N2 was digested with restriction enzymes *Eco*53KI-*Sph*I and ligated into *Sna*BI-*Sph*I digested pFastBacHA that resulted in a plasmid, pFastBacMHAN1N2, that encoded the HA, N1, N2 and M1 genes, with polyhedrin promoter and transcription termination sequences for each gene. For the co-expression of all four proteins, the individual gene segments were placed under the transcription control of the polyhedron promoter. SV40 poly (A) signal element was used for the termination of transcription and improving translational efficiency. Transformation of bacmid transfer plasmid (pFastBacMHAN1N2) containing four influenza genes into *E. coli* DH10Bac competent cells (Thermo Fisher Scientific, MA, USA) resulted in the generation of recombinant bacmid DNA.

Development and purification of VLPs. The recombinant bacmid DNA was transfected into actively growing Sf9 cells (Gibco, NY, USA) seeded in a 6-well plate using CellFectin reagent (Thermo Fisher Scientific, MA, USA). Transfection plates were incubated at 27°C and observed daily for seven days. The culture supernatant containing recombinant baculovirus was clarified by centrifugation for 5 min at 500 x *g* and passaged once in Sf9 cells to produce P1 working stock for VLP bulk production. VLPs obtained were purified through 20–60% discontinuous sucrose gradient ultracentrifugation.

Detection and quantification of VLPs. Purified VLP samples were adsorbed onto carbon-coated grids, stained with 2% phosphotungstic acid and visualized by using a transmission electron microscope (Philips, Columbus, OH). VLP protein

expression was verified using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. Total protein was estimated by bicinchoninic acid (BCA) method using Pierce™ BCA Protein Assay (ThermoFisher Scientific, MA, USA). HA and NA titers of VLPs were determined using two-fold dilutions of influenza VLPs in PBS. The hemagglutination unit (HAU) was determined as the highest dilution of VLPs showing visible agglutination of red blood cells. Neuraminidase activity of VLPs was assessed using substrate 20-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (Sigma, MO, USA) following the protocol (https://www.isirv.org/site/images/stories/avg_documents/Methodology/munana_ic50_sop_for_external.pdf) provided by Health Protection Agency, London.

Immunogenicity testing. Four groups of inbred female BALB/c mice (7 per group) of age 6–8 weeks were injected intramuscularly, on days 0 and 21 in the left hind limb, with 5 µg or 10 µg of VLPs in PBS with and without Quil-A® adjuvant (InvivoGen, CA, USA). Quil-A is a saponin vaccine adjuvant, which combined with cholesterol and phospholipids forms immunostimulatory complexes (ISCOMs) that can activate both cell-mediated and antibody responses. The dosing concentration used was 15 µg per mouse as prescribed by the manufacturer. The normal control group (7 mice) received only PBS, and the adjuvant control group (7 mice) received 15 µg of Quil-A adjuvant in PBS. 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, 14, 35 and 56 for serological analysis.

Antibody responses to influenza VLPs. Antibody responses to influenza VLPs and VLP + adjuvant was assessed by hemagglutination inhibition (HAI) antibody assay and neuraminidase inhibition (NAI) antibody assay. A/California/07/2009(H1N1) and A/Victoria/361/2011(H3N2) viruses used for the assays are egg-grown viruses that have been partially purified and provided in the Reagents for the typing of human influenza isolates kit (WHO collaborating center for reference and research on influenza, Melbourne, Australia). HAI assay was performed by treating serum samples with receptor-destroying enzyme (RDE) and serially diluted twofold in microtiter plates. An equal volume of (4 HAU/25 µl) influenza A/California/07/2009(H1N1) virus antigen was added and incubated followed by the addition of 0.5% chicken RBCs. Reciprocal of the highest dilution of the serum showing non-agglutinated RBC was considered as HAI antibody titer.

NAI antibody titer was measured by enzyme-linked lectin assay performed according to the published protocol. (Couzens *et al.*, 2014) NAI antibody titers were determined against both A/California/07/2009 (H1N1) and A/Victoria/361/2011(H3N2) viruses detected. Reciprocal of the highest dilution of serum resulting in at least 50% inhibition of NA activity was considered as NAI antibody titer.

Results

Analysis of recombinant bacmid

PCR was performed using pUC/M13 primers to confirm that the MHAN1N2 DNA was transposed into the bacmid. The primers were directed at sequences on either side of the mini*att*Tn7site within the *lac*Zα-complementation region of the bacmid and a product of size ~10, 000 bp (Fig. 1a) was obtained, indicating successful transposition of DNA into bacmid.

Transfection of Sf9 cells

On day 7, the transfected cells displayed CPE. In comparison with the negative control well (no recombinant DNA), the test well had enlarged cells with poor adherence to the substrate and low cell density indicating successful transfection (Fig. 1b).

Detection and quantification of VLPs

VLPs, imaged by transmission electron microscopy, were 100 nm in size, spherical, enveloped with protein spikes, presumed to be HA and NA glycoproteins projecting from the surface of each particle (Fig. 2a). The western blot analysis of proteins separated on SDS-PAGE and probed with anti-serum detected bands of the size 70 kDa, 55 kDa, and 27 kDa, respectively, demonstrating simultaneous and independent expression of all the recombinant influenza virus proteins namely HA, NA, and M, respectively (Fig. 2b). The total protein amount was estimated to be 1 mg/ml by the BCA method. HA activity was determined as 256 HAU/25 µg of VLPs and the highest dilution showing agglutination was 1:256. The neuraminidase activity of VLPs estimated using MUNANA as substrate was 320 RFU/25 µg of VLPs and the highest dilution with NA activity was 1:4096.

Antibody responses to VLPs

Serum samples collected on days 14, 35 and 56 were tested using NAI and HAI assays to measure antibody responses to VLPs. Anti-NA responses were evaluated using influenza A/California/07/2009(H1N1) virus and A/Victoria/361/2011(H3N2) virus (Table 1). Pre-immune sera from mice had no detectable specific anti-NA/HA antibodies.

On day 14, titers of anti-NA antibodies were low in all four test groups. A two to four-fold rise in anti-NA antibody titers was observed in the sera collected on day 35 in comparison to day 14 in all test groups. The number of responders in all groups on day 35 was up to 90–100%,

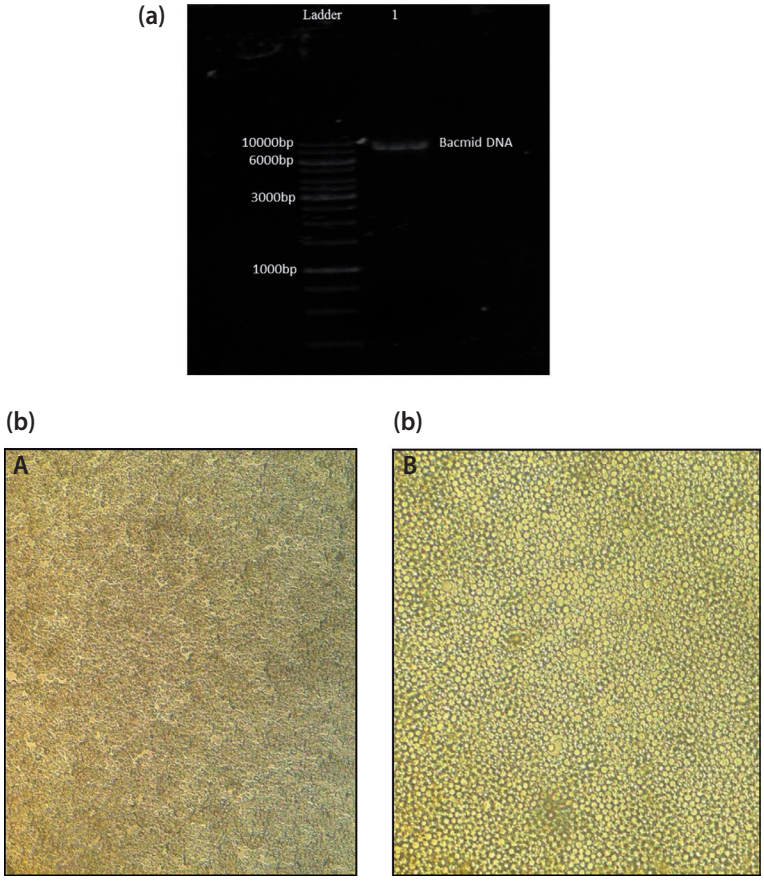

Fig. 1

Analysis and transfection of recombinant bacmid
(a) Gel picture of PCR amplified bacmid DNA. Lane 1 shows a product of size ~10, 000bp. **(b)** Cytopathic effect of recombinant baculovirus **(A)** Negative control well **(B)** Test well showing signs of virus growth with enlarged round cells on day 7.

except in 5 µg VLPs + 15 µg adjuvant (36%). Mice that received 5 µg VLPs and 5 µg VLPs + 15 µg adjuvant showed an increase in anti-NA antibody titers between sera collected on day 35 and day 56. The anti-NA antibody titers in the mice group injected with 10 µg VLPs showed a significant increase by day 35, however, titers decreased by day 56 (Fig. 3). Mice injected with 10 µg VLPs + 15 µg adjuvant showed high titers of anti-NA antibodies of 1977 and 457

Table 1. Neuraminidase and hemagglutination inhibition antibody assay

VLP (Dose)	Quil-A (Dose)	Average NAI antibody titer against A/California/07/2009(H1N1)			Average NAI antibody titer against A/Victoria/361/2011(H3N2)			Average HAI antibody titer against A/California/07/2009(H1N1)		
		Day 14 (responders)	Day 35 (responders)	Day 56 (responders)	Day 14 (responders)	Day 35 (responders)	Day 56 (responders)	Day 14 (responders)	Day 35 (responders)	Day 56 (responders)
5µg	-	11 (1/7)	92 (6/7)	211 (7/7)	11 (2/7)	97 (7/7)	120 (7/7)	7 (5/7)	7 (5/7)	16 (6/7)
5µg	15µg	17 (2/7)	92 (2/7)	171 (6/7)	34(4/7)	80 (3/7)	197 (7/7)	6 (4/7)	10 (4/7)	33 (7/7)
10 µg	-	3 (1/7)	274 (6/7)	206 (6/7)	11(2/7)	211 (6/7)	140 (6/7)	3 (2/7)	9 (5/7)	13 (7/7)
10 µg	15µg	11 (2/7)	1977 (7/7)	1223 (7/7)	34 (4/7)	457(7/7)	1149 (7/7)	4 (3/7)	59 (6/7)	57 (7/7)
-	-	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	8 (6/7)	10 (7/7)	10 (7/7)
-	15µg	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	0 (0/7)	7 (6/7)	10 (7/7)	10 (7/7)

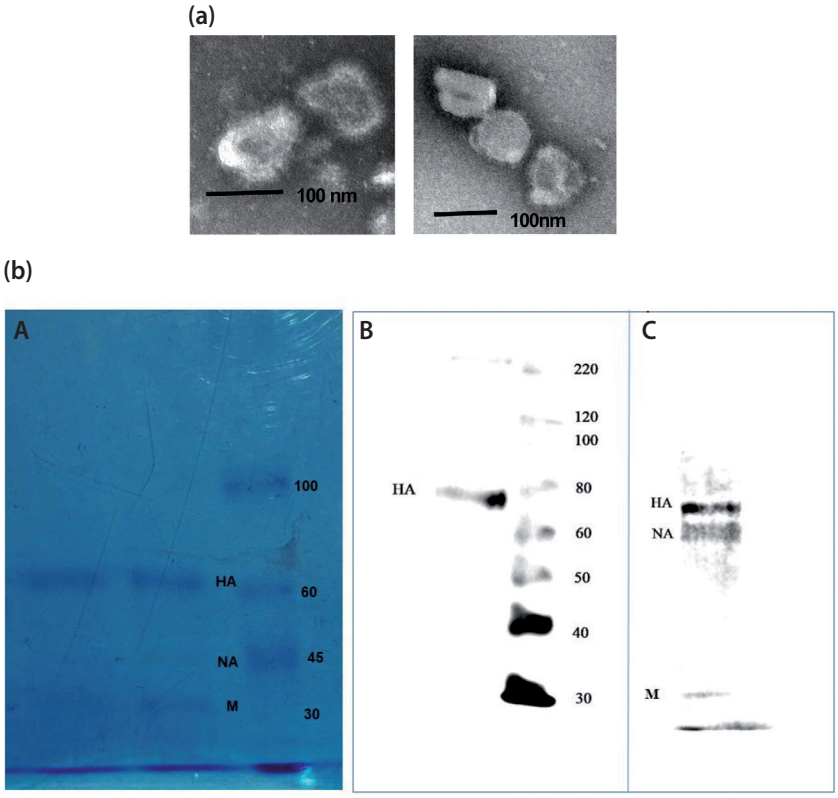

Fig. 2

Characterization of purified VLPs

(a) Electron micrograph of negatively stained influenza VLPs. Bar indicates the size in nm. **(b)** Determination of recombinant proteins. **(A)** SDS-PAGE and Coomassie brilliant blue stained gel. **(B)** Western blot analysis of VLPs probed with anti-HA antibodies (**C**) Western blot analysis of VLPs probed with influenza A polyclonal antibody.

against influenza A/California/07/2009(H1N1) and A/Victoria/361/2011(H3N2) virus, respectively on day 35. On day 56, a more than two-fold rise in anti-NA antibody titers (1149) was observed in the same group (10 µg VLPs + 15 µg adjuvant) against A/Victoria/361/2011(H3N2) virus, however, titer against A/California/07/2009(H1N1) virus was reduced (Fig. 3). Anti-HA antibody titers (Table 1) were low (≤10) compared to anti-NA antibody titers for all groups on day 35 except for the group injected with 10 µg VLPs + 15 µg adjuvant (59 HAI titer). There was no significant change in anti-HA antibody titers between day 35 and day 56 (Fig. 3). A background HA antibody titer of ≤10 was observed in the normal control and adjuvant control group.

Discussion

Egg-based production of influenza vaccines has 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, the need for faster, high-yielding techniques producing safer vaccines has been quintessential. Insect cell-derived vaccines are considered a new alternative to conventional egg-based influenza vaccines. The insect expression system offers several advantages in comparison to egg-based vaccines such as ease of handling, amplified yield, reduced manufacturing time, safety, and cost-effectiveness (Krammer and Grabherr, 2010). The baculovirus expression vector systems (BEVS) have been used for almost three decades to produce recombinant proteins in insect cells (van Oers *et al.,* 2015). The BEVS have also been used for influenza virus VLP production with considerably promising results. The VLP-based influenza vaccines have been shown to induce significant amounts of anti-HA and anti-NA antibodies in immunized mice (Pushko *et al.,* 2005; Bright *et al.,* 2007; Behzadian *et al.,* 2013; 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 the influenza virus namely, H1, N1, and M from influenza A (H1N1) virus and N2 protein from influenza A (H3N2) virus. HA is the major surface protein of the 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 analysis of NA gene sequences of influenza A (H1N1) and (H3N2) viruses strengthens the fact that genetic variations in these genes occur at a slower rate (Jagadesh *et al.,* 2016b, 2017), thus anti-NA antibodies can provide long-term protection. Based on this finding, to further increase the spectrum of protection conferred by the influenza VLPs, both N1 and N2 proteins were incorporated. Nevertheless, a strong N1- and N2-based immunity might be beneficial at the onset of 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 for the generation of influenza VLPs, therefore the M protein was also included in the VLP cassette (Gómez-Puertas *et al.,* 2000).

The constructed recombinant baculovirus DNA was used for the transfection of Sf9 cells and the production of VLPs. Thompson *et al.* (2015) assessed the expression of influenza VLPs in Sf9 and HEK293 expression systems and reported that VLPs production was 35 times higher in Sf9 cells than in HEK293 cells. HA activity was found higher and VLPs had a more homogenous morphology in Sf9 cells. VLPs produced using the insect cell culture system were purified by sucrose gradient ultracentrifugation and, upon electron microscopy and western blot analysis, the expression of HA, NA and M proteins was confirmed. Currently, there are no total particle quantification methods for influenza VLPs other than antigen-based methods such as HA assay, NA assay, and single radial immunodiffusion (Thompson *et al.,* 2013). In our study, the total VLP protein concentration estimated by the BCA method was 1 mg/ml, the HA activity was 256HAU/25 µl and the highest dilution of VLPs with NA activity was 1:4096. Single radial immunodiffusion could not be performed due to the unavailability of homologous reference HA antigens.

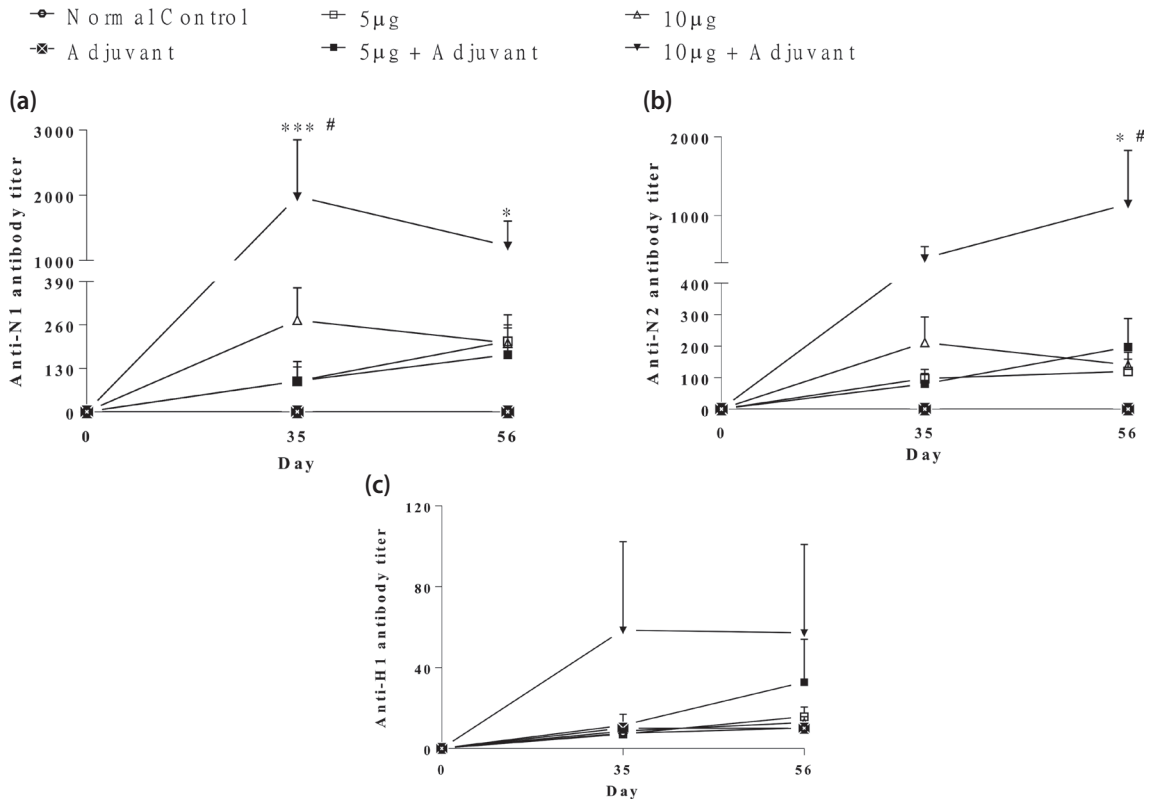

Fig. 3

Immune response elicited by influenza VLPs

Mice (n = 7) were administered influenza VLPs with/without adjuvant, or PBS per se, via intramuscular injection on days 0 and 21. Serum of each group was tested for anti-NA **(a&b)** and anti-HA **(c)** antibodies by NAI and HAI assay. Pre-immune sera from mice had no detectable specific anti-NA/HA antibodies. *p <0.05 & ***p <0.001 compared to normal control; #p <0.01 compared to 10 µg VLP.

The VLPs developed in this study were tested for immunogenicity by injecting varying concentrations (5 µg and 10 µg) with and without Quil-A adjuvant in a mouse model. Quil-A is derived from an aqueous extract from the tree *Quillaja saponaria*. Quil A has been used successfully for veterinary applications but is generally considered too toxic for human use. Reverse chromatography purified fractions from Quil-A, mainly QS-21, have been studied as an alternative to alum, especially when a strong vaccine-induced cellular response is required (Rajput *et al.*, 2007). QS-21, developed by GlaxoSmithKline, has been used for the development of adjuvant systems (AS), AS01 and AS04, which have been used for vaccine trials for HIV and HPV (Baz Morelli *et al.*, 2012). QS-21 adjuvant was not available for use in India during the initiation of this study in late 2017, thus Quil-A adjuvant was used for the animal study.

The VLPs with and without adjuvant induced a significant amount of anti-NA, but low anti-HA responses in mice. Invariably, a background HA antibody titer of ≤10 was observed in normal and adjuvant control groups. This could be due to the interference by specific inhibitors present in murine sera with the HAI assay (Cwach *et al.*, 2012). Increased anti-NA responses may be attributed to the presence of two NA proteins (N1 and N2) in VLP. High anti-NA antibody titers were induced with 10 µg VLPs + 15 µg adjuvant, indicating that Quil –A and its purified forms can be considered as adjuvants for influenza VLP-based immunogenicity studies. Induction of anti-NA immune responses in mice strengthens the premise that VLP-based vaccines containing NA are promising influenza vaccine candidates for inducing long-term immunity. Further studies incorporating an equal number of HA and NA proteins in influenza VLP will provide better insights into the anti-HA immune responses. The positive results obtained in this study serve as a platform for future studies with different combinations of influenza surface proteins in different animal models.

Ethical clearance

The study was initiated after approval was received from the Institutional Animal Ethics Committee (IAEC/KMC/29/2017).

Conflict of Interest. The authors declare no conflict of interest. The invention and the process have been filed for an Indian patent (Application No. 201741038086).

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