Evaluation of the antigenic stability of influenza virus like particles after exposure to acidic or basic pH

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Purpose: Virus-like particles (VLPs) are being developed as a promising vaccine platform and therapeutic delivery. Various strategies for effectively constructing VLPs have been studied, but relatively few studies have been done on various factors affecting storage. In this study, we investigated the antigenic changes of VLPs in an acidic or basic pH environment using influenza VLPs as an experimental model.

Materials and Methods: Influenza VLPs containing hemagglutination and M1 proteins were generated and their antigenicity and protective immunity *in vitro* and *in vivo* were evaluated after exposure to acidic (pH 4 and 5) or basic (pH 9 and 10) pH buffers.

Results: VLP exposed to basic pH showed similar levels of antigenicity to those stored in neutral pH, while antigenicity of VLP exposed to acidic pH was found to be significantly reduced compared to those expose neutral or basic pH. All groups of mice responded effectively to low concentrations of virus infections; however, VLP vaccine groups exposed to acid pH were found not to induce sufficient protective immune responses when a high concentration of influenza virus infection.

Conclusion: In order for VLP to be used as a more powerful vaccine platform, it should be developed in a strategic way to respond well to external changes such as acidic pH conditions.

Keywords: Virus-like particles, pH, Antigenicity, Protective immunity

Introduction

Egg-based inactivated vaccines have been used to prevent influenza, but recently various forms such as cell culture vaccines and live attenuated vaccines have been developed and commercially available [1,2]. Currently, development of subunit vaccines in the form of recombinant proteins expressed in prokaryotic and eukaryotic cells has been accelerated [3-5]. Virus-like particles (VLPs) used in this study are recombinant proteins expressed in insect cells and are being studied as effective vaccine platforms due to their high structural similarity to native viruses [6-8].

A typical example of successful commercialization of the VLP vaccine is the human papillomavirus (HPV) vaccine, which is composed of VLPs produced through self-assembly without lipid envelop using *Escherichia coli* or yeast expression system [9,10]. Although many other vaccines in the form of VLP were expected to be developed due to the success of the HPV vaccine, they are still in the research and development or clinical trial stage due to low yield during production and significantly reduced stabili-

ty in storage [11-14].

While some studies have been reported on the effect of pH, ionic strength, and temperature on the assembly of VLPs *in vitro* [15-20], few studies have been conducted on the effect of pH in the environment for storing of VLPs in relation to the vaccine efficacy. Since VLP vaccines are protein complexes produced through the assembly of recombinant proteins, the impact on storage environments such as pH is expected to be significant. In this study, we examined changes in physicochemical properties and immunogenicity of VLP exposed to various pH conditions using the influenza VLPs as a study model.

Materials and Methods

Virus, hemagglutination and M1 gene
Influenza A/PR8/34 virus (H1N1) was propagated in embryonated chicken eggs. Viral RNAs were extracted from allantoic fluids using Viral Gene spin (iNtRON Biotechnology Inc., Seoul, Korea) and the cDNA of hemagglutination (HA) and M1 genes were produced by reverse transcription-polymerase chain reaction (RT-PCR) using PrimeScript One Step RT-PCR kit ver2 (TaKaRa, Dalian, Japan). The HA and M1 genes were cloned into pFastBac1 and then transformed into DH10Bac for generation of bacmids. The bacmids were transfected into *Spodoptera frugiperda* (sf9) cells using lipofectamine agent (Invitrogen, Waltham, MA, USA) and incubated at 27°C for 3 days to obtain recombinant HA and M1 baculoviruses, respectively.

Production and purification of influenza PR8 virus-like particles
VLPs of influenza A/PR/8/34 (H1N1) were expressed as previously described [21]. Briefly, recombinant baculoviruses encoding HA and M1 of A/PR/8/34 were co-infected into sf9 cells at a multiplicity of infection of 0.5 to 1. Culture supernatants were harvested 72 hours post-infection by centrifugation at 4,000 rpm for 15 minutes. Then the supernatants were concentrated by stirred ultrafiltration system using stirred cell (Millpore 5124) with 100 kDa molecular weight cut-off membrane. After concentration, PR8 VLPs were purified by sucrose gradient purification at 30,000 rpm for 1 hour at 4°C. The protein concentration of VLPs were determined by BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA; 23225). The electron microscopic picture of PR8 VLPs was taken as described previously [22].

Exposure of PR8 virus-like particles to various pH buffers
Five different pH buffers (pH 4, pH 5, pH 7, pH 9, pH 10) were produced by adding aqueous solution of sodium hydroxide (NaOH) and hydrochloric acid (HCl) to distilled water. Influenza PR8 VLPs were diluted in five different pH buffers and incubated for 1 hour or 4 hours at 25°C.

Hemagglutination assay
HA assay was performed to determine HA titers of VLPs under each pH condition. Detailed procedure of HA assay was previously described [23]. PR8 VLPs were serially two-fold diluted in phosphate-buffered saline (PBS) and incubated with 0.5% chicken red blood cells at 4°C for 30 minutes.

Western blot
Western blot analysis was carried out to confirm the expression of HA and M1 of PR8 VLPs. A changed features of the VLPs caused by incubation with pH buffers for 4 hours at 25°C were also analyzed by Western blot. The VLPs were separated by SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis) using 12% acrylamide gel and transferred to PVDF (polyvinylidene fluoride) membrane by semi-dry transfer system. The HA and M1 were detected by mice immunized sera with PR8 inactivated virus.

Mice immunization and virus challenge
Six-week-old female BALB/c mice (n=10) were immunized intramuscularly with a single dose of purified PR8 VLPs (2.5 µg) which were exposed under five different pH buffers for 4 hours at 25°C. The mice were challenged with sub-lethal or lethal dose of A/PR/8/34 live virus by intranasal injection after 4 weeks from the vaccination as described previously [24]. Body weight and survival rate of challenged mice were monitored every day for 13 days.

Enzyme-linked immunosorbent assay
After 3 weeks from vaccination, blood samples were collected by retro-orbital bleeding and stored at -20°C. Specific immunoglobulin G (IgG) antibody response to PR8 VLPs was determined by enzyme-linked immunosorbent assay (ELISA) as previously described [25]. ELISA plates (96-well) were coated with 1 µg/mL PR8 inactivated virus at 4°C overnight. Plates were blocked with 3% BSA in PBS at 37°C for 1 hour 30 minutes. Two-fold diluted serum samples were added to the plate and incubated at 37°C for 1 hour 30 minutes. HRP-conjugated goat anti-mouse IgG antibody was used as secondary anti-

body. For development, 50 μL TMB (3,3′,5,5′-tetramethylben-zidine) solution was added to the plate. Then 50 μL of 0.5 M sulfuric acid was added as stop solution. The optical density was measured at 450 nm and determined using ELISA reader.

Hemagglutination inhibition assay

For determination of PR8 VLP specific quantitative antibodies, HA inhibition assay was conducted as previously described [26]. To remove nonspecific inhibitors of HA, sera were treated with receptor-destroying enzyme (RDE II; Denka Seiken, Tokyo, Japan) at a ratio 1:3 at 37°C for 20 hours followed by inactivation step at 56°C for 30 minutes. Sera with RDE were tested at an initial dilution 1:8 in PBS with following two-fold serial dilution. The amount of PR8 inactivated virus equivalent to four HA units was added followed by incubation at 25°C for 1 hour. Chicken red blood cells (1%) were added and incubated at 4°C 30 minutes.

Statistics

All parameters were recorded for individuals within all groups and statistical analyses were performed with Graph-Pad Prism Software ver. 5.0 (GraphPad Software, San Diego, CA, USA). For evaluating the statistical differences among groups, Student t-test was performed and p-values of less than 0.05 were considered statistically significant.

Ethics statement

The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee in Sungshin University (IACUC approval no., SSWIACUC-2020-006).

Results

Production of the influenza PR8 virus-like particles and exposure to basic and acidic pH

Influenza PR8 VLPs were generated by co-infecting sf9 cells with baculoviruses expressing influenza M1 and HA proteins derived from influenza A/Puerto Rico/8/34 (H1N1) (Fig. 1A). Morphology of VLPs was confirmed by the electron microscopic investigation of the negatively stained samples (Fig. 1B) and Western blotting was performed to confirm the expression of HA and M1 in VLPs (Fig. 1C, D). To expose the VLPs to various pH conditions, VLP samples were exposed to acidic (pH 4 and 5), neutral (pH 7), and basic (pH 9 and 10) pH, respectively and maintained at room temperature for 1 hour and 4 hours (Fig. 2). Visual confirmation of changes in physical properties of the VLPs after the exposure period showed no clumps or abnormal precipitate.

Effects of pH on the antigenic stability of virus-like particles *in vitro*

The antigenic stability of VLP samples exposed to basic, acidic, and neutral pH were tested *in vitro*. Cleavage patterns of HA in the VLPs were determined by Western blotting. As shown in Fig. 3A, there was no significant change in HA cleavage pattern after exposure to any pH conditions. HA proteins maintained HA0 form that was not cleaved into HA1 and HA2 after expose to both acid and basic pH conditions in the absence of an additional protease. To determine the binding ability of the VLPs with receptor in cells, HA assay was performed with chicken red blood cells. At neutral pH, Influenza VLPs had
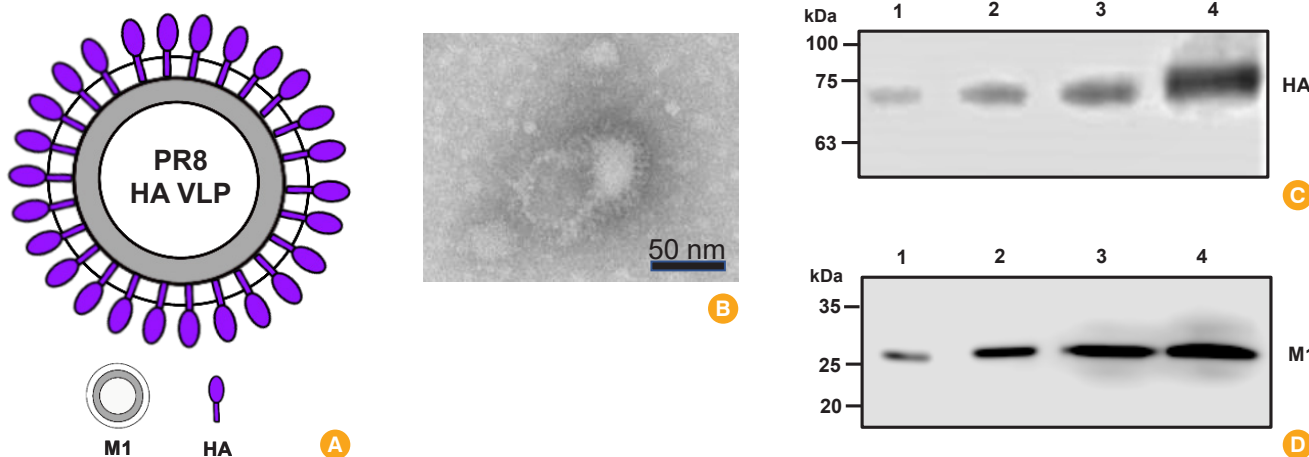

Fig. 1. Characterization of influenza PR8 virus-like particles (VLPs). (A) A schematic diagram of influenza PR8 VLPs expressing hemagglutination (HA) and M1 proteins illustrated above and (B) negative stain electron microscopy of the VLPs showed. (C, D) HA (75 kDa) and M1 (25 kDa) proteins in the VLPs were detected as indicated in Western blot analysis.

activity of 128 HA unit but HA activity was not detected when the VLPs were exposed to pH 4 for 1 hour (Fig. 3B). Exposure of the VLPs to pH 5 for 1 hour reduced HA activity by about half and leaving only about 16% HA activity during exposure of 4 hours (Fig. 3B, C). On the other hand, VLP samples exposed to basic pH did not show any changes in HA activity (Fig. 3B, C). These results showed that binding abilities of HA proteins, which play a crucial role in binding to cell receptors, were reduced significantly when stored or exposed at acidic conditions.

Effects of pH on the immunogenicity of virus-like particles *in vivo*

Female BALB/c mice were vaccinated with VLPs exposed to various pH to determine the changes in immunogenicity of VLPs. Sera were collected from all group of mice at 3 weeks after vaccination and HA specific IgG responses were measured by ELISA. Compared to neutral pH group, significant drop in antibody levels was observed in acidic pH group (pH 4), while there was no significant difference in antibody levels present in basic pH group (pH 9, 10) (Fig. 4A, B). These results were more clearly observed when HA inhibition assay was performed to measure the level of functional antibodies. VLP vaccines exposed to acidic pH did not induce sufficient neutralization antibodies, whereas VLP vaccines exposed to basic pH did not differ statistically from neutral pH, despite its slight decrease (Fig. 4C).
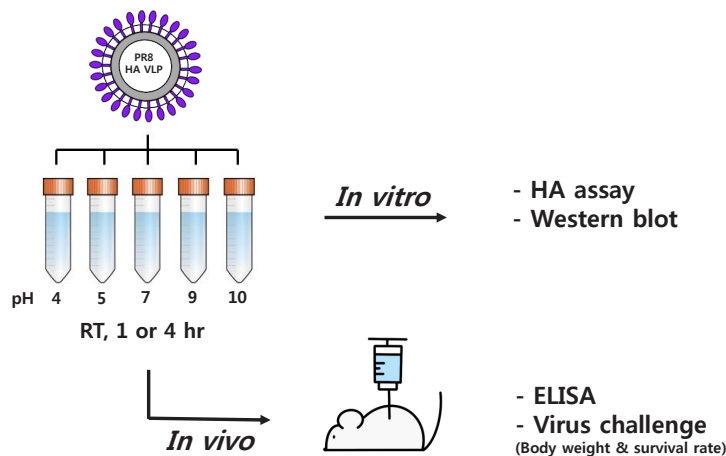

Fig. 2. Experimental design for measuring antigenic changes in the virus-like particles (VLPs) exposed to various pH conditions. Influenza PR8 VLPs were stored in a pH buffer corresponding to basic or acidic conditions at room temperature (RT) for 1 hour or 4 hours and *in vitro* antigenicity and structural integrity were measured by hemagglutination (HA) assay and Western blotting respectively, and *in vivo* immunogenicity was measured by animal experiments. ELISA, enzyme-linked immunosorbent assay.
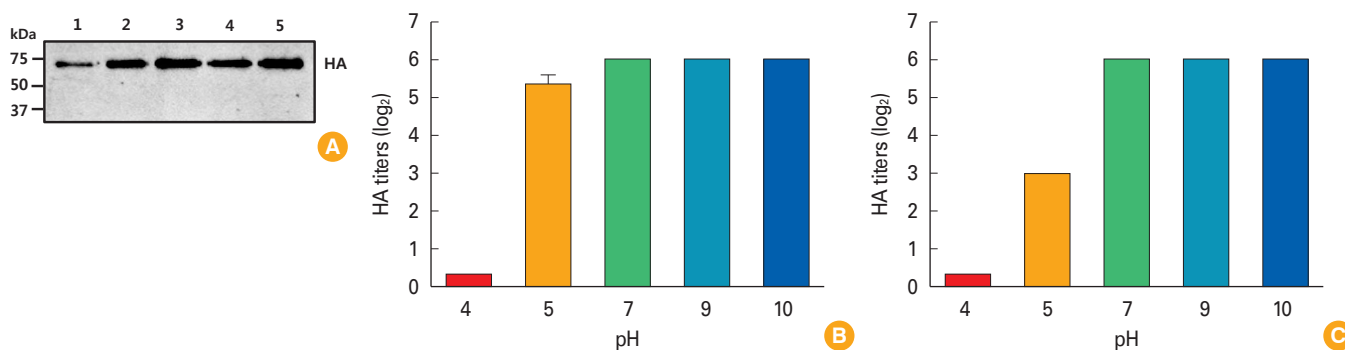

Fig. 3. Examination of structural integrity and hemagglutination (HA) activity of the virus-like particles (VLPs) exposed to various pH conditions. (A) Same amount of influenza PR8 VLPs were stored in different pH buffers and then Western blotting was performed using HA-specific antibodies. Similarly, same amount of the VLPs were stored in different pH buffers for 1 hour (B) or 4 hours (C) and then HA assay was performed to measure the specific binding activity between chicken blood cell and HA.
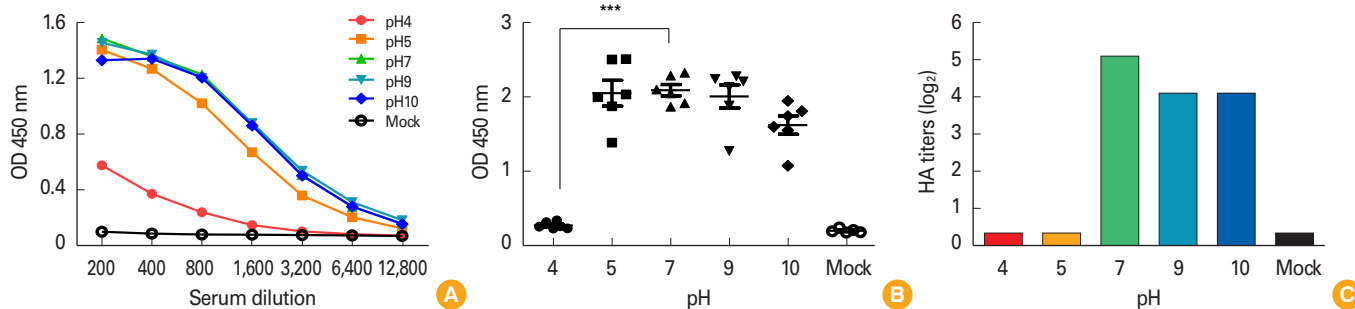
Fig. 4. Evaluation of antigenicity of the virus-like particles (VLPs) exposed to various pH conditions. Influenza PR8 VLPs were stored in different pH buffers for 4 hours, then inoculated with female BALB/c mice. Sera of the VLP immunized mice were collected 3 weeks after immunization and hemagglutination (HA) specific immunoglobulin G responses (A, B) and neutralizing antibodies (C) were determined by enzyme-linked immunosorbent assay and HA inhibition (HI) assay, respectively. OD, optical density. ***$p<0.001$; significant differences (by Student t-test).
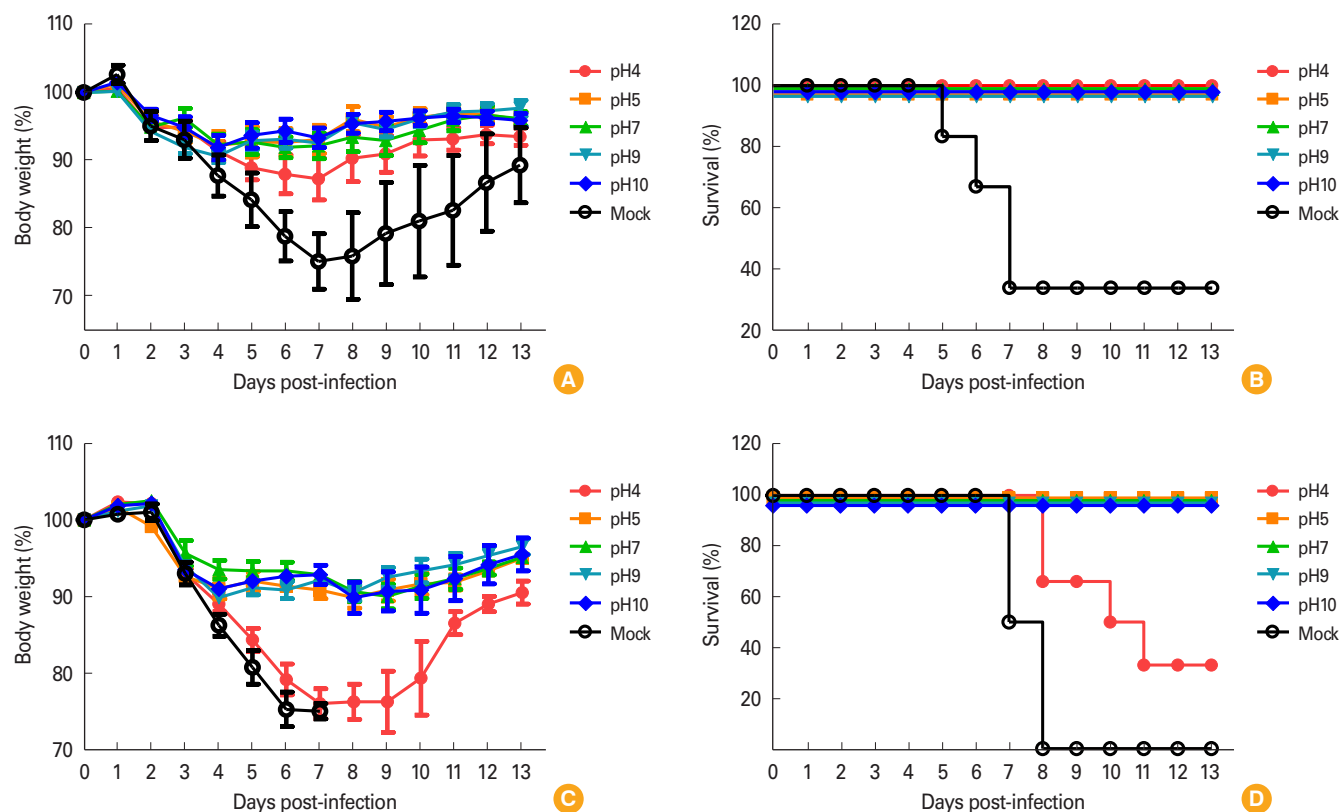

Fig. 5. Evaluation of protective immunity of the virus-like particles (VLPs) exposed to various pH conditions against influenza virus lethal challenge. Influenza PR8 VLPs were stored in different pH buffers for 4 hours, then inoculated with female BALB/c mice. At 4 weeks after immunization, six of immunized mice were infected with sub-lethal dose (1 MLD_{50}) (A, B) and four of immunized mice were infected with lethal dose (5 MLD_{50}) (C, D) of influenza A/PR/8/34 (H1N1) wild type virus by intranasal route. Mice were monitored for 13 days following influenza virus infection to determine bodyweight changes and survival rate.

Protective immunity of virus-like particles exposed to acidic or basic pH

To compare the differences in the protective immunity of VLP vaccines exposed to various pH environments, mice were infected with a sub-lethal dose (1 MLD_{50}) of influenza virus at 4 weeks after vaccination. In case of pH 4 group, there

was weight loss by about 5%, whereas no weight loss occurred in other groups. However, this weight loss is not as much as expected considering significant difference of antibody levels (Fig. 5A). In contrast, there was no difference in survival rates of all groups (Fig. 5B). On the other hand, infection with high dose of influenza virus (5 MLD_{50}) resulted in a

severe weight loss of about 25% and high mortality rate (75%) were observed in pH 4 groups. All other groups including neutral pH showed 10% weight loss and survived 100% (Fig. 5C, D).

Discussion

Previous studies in our group have shown that influenza VLPs have the most stable at pH 7.0 to 7.2, which corresponds to physiological pH under room temperature storage conditions, so neutral pH was set as the reference point in this experiment to examine the effect of pH. In the preliminary experiment, pH changes less than 1 log level from neutral were not significantly affected both *in vitro* and *in vivo*, so 1–3 log level variations were tested, and extreme low and high pH were not tested.

Of all pH environments applied to this study, the one that showed the greatest change in physical properties was pH 4, which corresponds to acidity. First, a slight change in the amount of native protein was observed in Western blots and a significant reduction in the cell binding capacity of VLPs was observed in HA assay. In contrast, VLPs exposed to basic conditions did not differ significantly compared to neutral pH conditions.

ELISA confirmed that HA protein, the most immunogenic antigen of influenza VLP vaccines, had significant changes in antigenicity when exposed to acidic pH. In addition, HA inhibition assay showed that exposure of VLP to low pH had a significant impact on inducing neutralizing antibodies.

HA proteins not only play a role in viral attachment to receptor in cell, but also in the uncoating of viruses within infected cells. During the uncoating process, structural deformation of fusion peptide is induced by a low pH environment, and this is an essential step for viruses to replicate within cells. This means that HA proteins are fundamentally designed to facilitate structural variation depending on pH conditions, and these properties are considered important factors when developing vaccines.

While the antigenicity of VLP exposed to acidic pH decreased significantly compared to basic and neutral pH, it did not result in a proportional reduction in protective immunity at sub-lethal dose infection. This can be explained by simultaneous effect of antibody responses and cellular immunity to inhibit virus proliferation in sub-lethal dose of virus infection. On the other hand, if the virus inoculum was high in the early stages of the infection failing to induce sufficient adap-

tive immunity, virus proliferation was not properly controlled, resulting in high mortality and weight loss.

When the vaccine exposed to each pH were vaccinated twice, all groups survived 100% without weight loss even at a high dose infection (data not shown). This result demonstrates that sufficient immune responses can be induced by boost shot even if antigenic change occurred in HA by acidic pH condition, and M1 protein may have contributed in part to inducing immunity against virus infection as well.

As a result, VLP vaccines, a type of recombinant protein vaccine, may require special care to avoid exposure to acidic pH environments, and the use of preservatives to offset these changes may need to be considered in future vaccine developments.

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