



Aerosol Particle Size Distribution and Genetic Characteristics of Aerosolized Influenza A H1N1 Virus Vaccine Particles

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ABSTRACT

Influenza has increasingly become a global issue that threatens human lives worldwide. While an aerosol-type influenza vaccine has been licensed for the treatment of seasonal influenza via intranasal administration, the physical and genetic characteristics of aerosols containing vaccine particles have not yet been reported in either a native or artificial environment. In this study, we aerosolized a conventional vaccine solution containing inactivated split influenza particles and measured the size distribution of the aerosol particles after nebulization. We also tested a novel method for detecting a minimal amount of the viral particles in the aerosols by amplifying the viral genomic RNAs in the vaccine solution via reverse transcription-PCR. In the results, we found via TEM that the morphology of the viral particles in the vaccine solution was not significantly deteriorated by the inactivation process. The aerosolized vaccine particles exhibited a mode diameter of 130 nm. In addition, the viral RNAs were successfully amplified from the inactivated split virus vaccine solution even after the nebulization process. Taken together, the current experimental results provide basic information regarding the general characteristics of the inactivated influenza viral particles in the vaccine, including genetic properties, and may contribute to the effective use of the vaccine solution in medical protocols.

Keywords: Influenza A H1N1; Influenza; Bioaerosol; Vaccine; RT-PCR.

INTRODUCTION

The pandemic outbreak of H1N1 flu due to the influenza A H1N1 virus occurred in over 130 countries and resulted in over 10,000 deaths worldwide (European Centre for Disease Prevention and Control, 2010). The Centers for Disease Control and Prevention in the U.S. estimates that around 55 million people were infected with 2009 H1N1, resulting in 246,000 H1N1-related hospitalizations in the U.S. (CDC, 2010). These outbreaks of H1N1 flu had an enormous impact on the transportation industry, health-related businesses, and increased public concerns over air infection. The flu, including H1N1 flu, is spread from person to person

via airborne transmission of influenza virus bioaerosols (Salgado *et al.*, 2002; Tellier, 2006; CDC, 2010), where the term of bioaerosol is defined as aerosol of biological origin and receives an attention due to relation with air infection (Grinshpun *et al.*, 2010; Kumagai *et al.*, 2010). The influenza virus is a lipid-enveloped ribonucleic acid (RNA) virus containing segmented negative-sense genomes. Influenza viruses can be transmitted in three ways: aerosols, large droplets, and direct contact with secretions. The recent worldwide pandemic spread of H1N1 flu along with reviews of studies on influenza viruses (Tellier, 2006) provides evidence that aerosols are the dominant route for transmission of influenza A H1N1 viruses.

A vaccine is a biological substance that induces protection against a particular disease. Vaccines are frequently prepared from weakened or inactivated forms of a disease-causing microorganism. Vaccine administration (Vaccination) may be oral, intranasal with spray, or via injection with needles. Among these methods, intranasal administration, in which aerosolized vaccines are delivered

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through nose cavities, has drawn the attention of researchers as one of the most effective means for controlling diseases resulting from viral infection, especially in response to the increased demand for mass vaccination at relatively low cost compared to typical injection methods. In addition, intranasal vaccines have been shown to be significantly more effective than injection ones in inducing a mucosal immune response in response to influenza virus, as the portal of the virus entry is the mucosa layer that lines the respiratory tracts exposed to the external environment (Muszkat *et al.*, 2003). Since several problems have been reported in using weakened live virus vaccine against influenza, including side effects in immune-compromised patients and the potential for reversion to pathogenic strains, many studies have suggested that intranasal delivery of inactivated influenza virus vaccine is a logical alternative (Smith *et al.*, 2003). Furthermore, successful delivery of several other aerosolized viral vaccines through the respiratory tract has recently been reported (Corbett *et al.*, 2008; Diaz-Ortega *et al.*, 2010). Despite the increasing need for optimal intranasal delivery of conventionally prepared vaccines, the physical and genetic characteristics of aerosols containing virus particles generated from vaccines have not been reported yet.

In this study, we aerosolized influenza A H1N1 virus vaccine particles and measured aerosol particle size distribution using a scanning mobility particle sizer (SMPS) and a particle size distribution analyzer (PSD). To examine the physical shape and size of vaccine particles containing influenza A H1N1 virus, we used transmission electron microscopy (TEM), a well-established and widely used technique for assessing the size and morphology of virus particles. Also, we developed a new strategy for the detection of vaccine particles based on polymerase chain reaction (PCR) with reverse transcription (RT-PCR) of a gene of influenza A H1N1 virus, and successfully used it to identify vaccine particles in the initial vaccine solution as well as in the collected aerosols after nebulization. The data obtained from this study may provide valuable information for the efficient administration of an intranasal influenza vaccine and improved aerosolized vaccination protocols.

MATERIALS AND METHODS

Aerosolization

Fig. 1(a) shows a schematic diagram of the experimental aerosolization setup. The aerosolization system comprised a compressed air tank, a HEPA filter, a mass flow controller, a nebulizer, and diffusion dryers. To aerosolize the vaccine particles, we prepared several liquid suspensions with varying dilution ratios. We mixed vaccines with different amounts of filtered sterile water, yielding four types of suspensions: A (vaccine 1: water 29), B (vaccine 1: water 59), C (vaccine 1: water 119), and D (filtered sterile water). We then poured the diluted suspensions into a nebulizer for each experiment. For the genomic tests, we prepared a particular solution which was produced by mixing 0.5 mL of a vaccine with 26.5 mL of filtered sterile water (similar

to solution B), due to technical reasons particular to the instruments. The nebulizer was used to spray the liquid suspension as small droplets containing the vaccine particles. The nebulizer used was a Microbiological Research Establishment Type-6 Jet Collision Nebulizer (Collision MRE 6 Jet, BGI Collision Nebulizer, BGI Inc., MA, USA). Aerosolized vaccine particles were passed through two diffusion dryers in order to remove moisture, after which they were diluted by additional airflow of dry, filtered, and compressed air at a rate of 4 L/min. We used a particle size distribution analyzer (PSD 3603, TSI Inc., MN, USA) and a scanning mobility particle sizer (SMPS, TSI Inc., MN, USA; DMA 3081; CPC 3025) to measure the aerodynamic particle size distribution of the aerosolized particles. The PSD 3603 has effective measurement size range from 0.5 μm to 100 μm , whereas the SMPS covers particles ranging from 10 nm to 700 nm in size. For the genomic experiments, aerosolized vaccine particles were sampled with a BioSampler (SKC Inc., PA, USA), which is operated using vacuum pumps equipped with flow meters (GAST IAQ Pump, EMS Inc., SC, USA). The aerosolized vaccine particles were collected in 20 mL of sterile water at a nominal flow rate of 12.5 L/min for 10 min for the genomic experiments. Finally, the sampled suspensions generated by the BioSampler were plated onto test plates and tubes.

Vaccine Solution

The vaccine solution (VAXIGRIP Vaccine; SANOFI Pasteur S.A., Lyon, France) was the material used to generate vaccine particles in this experiment, and was a sterile trivalent vaccine prepared from influenza viruses (A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Brisbane/60/2008). The vaccine was prepared from virus split by Triton X-100, inactivated by formaldehyde and diluted in phosphate buffered saline solution to the required concentration. No adjuvant or preservative was added.

Transmission Electron Microscopy

To determine the physical shape and size of vaccine particles containing influenza A H1N1 virus, the particles in vaccine solution were examined by TEM (Model-7650, Transmission Electron Microscopy, Hitachi, Japan). For negative staining, the vaccine solution was applied to carbon-coated formvar on copper grids, then allowed to dry and adhere for 5 min. The excess fluid was then drained by touching the edge of the grids with a torn piece of filter paper (Whatman, No. 1). Staining solution (0.2% uranyl acetate, Electron Microscopy Science, USA), was added to samples on the grids. After 2 min, the excess staining solution was drained, and the prepared samples were examined immediately (Hayat and Miller, 1990).

Genetic Analysis of Vaccine Particles (Reverse Transcription and Polymerase Chain Reaction)

Fig. 1(b) shows a schematic diagram of the genetic analysis of vaccine particles. To investigate the presence of influenza A H1N1 virus genes in collected aerosols after nebulization as well as in the initial vaccine solution, we

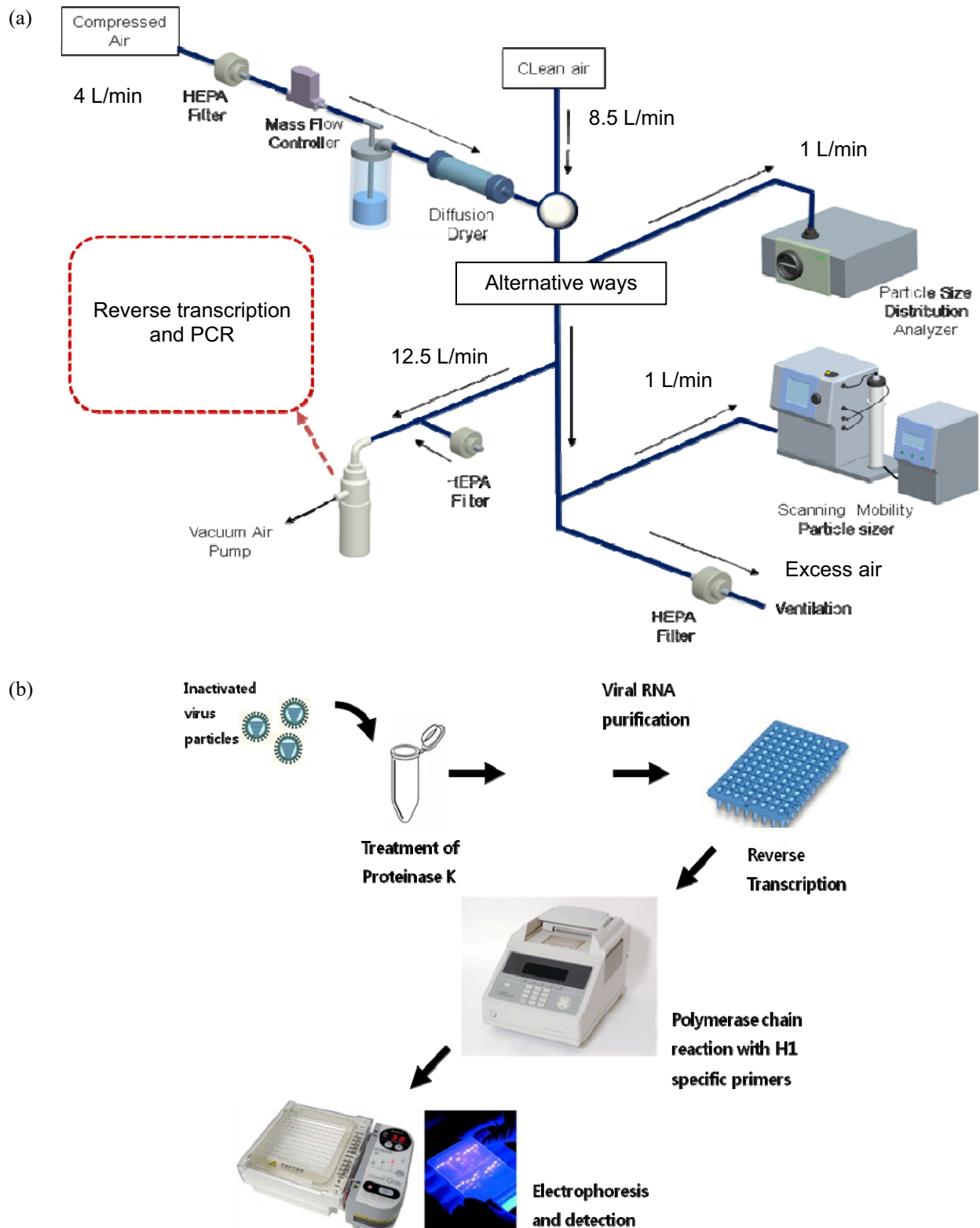


Fig. 1. Schematic diagram of the experimental setup. (a) Aerosol measurement systems, (b) Genetic analysis system (reverse transcription and PCR).

performed PCR to detect viral genomic RNA. In the experiment, 500 μL of the vaccine solution with 26.5 mL of filtered sterile water was nebulized, after which the aerosol particles were collected and transmitted to 15 mL

of digestion buffer (200mM Tris-HCl, 1.5 mL MgCl_2 , 2% SDS, pH 7.5). To detect viral genomic RNA, one tenth of the collected aerosol suspensions were treated with Proteinase K (10 $\mu\text{g}/\text{mL}$) for 1 h at 45°C, after which the

RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform. Proteinase K digests viral proteins that are tightly bound to viral RNA genomes, thus making it easy to purify RNAs. After precipitation with ethanol, the RNA pellet was dissolved in RNase-free water (Masuda *et al.*, 1999). For the detection of genomic RNA, we first synthesized complementary deoxynucleic acids (cDNA) via reverse transcription reaction. One tenth of the precipitated RNAs were reverse-transcribed with random primers according to the manufacturer's protocol (Maxime RT PreMix kit; iNtRON Biotechnology, Sungnam, Korea), followed by PCR amplification using a set of oligonucleotides as primers of the DNA synthesis reaction. The two primers were designed for specific binding to the Hemagglutinin gene sequence (H1) of the vaccine strain. The first nucleotides of the two primers were positioned at nucleotides 781 and 897, respectively, from the 5' end of the genomic fragment containing the H1 gene. Thus, the expected size of the amplified fragment was 116 nucleotides long. The sequences were 5'-TTGAACCCGGGGATACAATAA -3' for the forward reaction and 5'-TCCATTGGTGCATTTGAGTTG -3' for the reverse reaction. In the experiment, viral RNAs from the two solutions, the initial vaccine solution and the collected aerosols, were analyzed. For the negative controls, equal amounts of each of the two solutions were also amplified without reverse transcription. PCR reaction mixtures included 2 μ L of 10X buffer, 0.8 μ L of $MgCl_2$ (25 mM), 2 μ L of dNTP (0.2 mM), 2 μ L of primer (20 pM), 15U of Taq polymerase, and 1 μ L of cDNA in a reaction volume of 20 μ L. The temperature cycle program consisted of one cycle at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 30 sec. The final extension was at 72°C for 5 min, after which the reaction was cooled to 4°C. The PCR products were visualized on a 1.5% agarose gel and were verified by sequencing. A 100 bp DNA marker was used for agarose gel electrophoresis.

RESULTS AND DISCUSSION

Morphology of Inactivated Influenza Viral Particles in TEM Images

Fig. 2 shows images of particles from the vaccine solution taken by TEM. Currently, several TEM images showing actual live influenza viruses including H1N1 have already been reported (Nayak *et al.*, 2004; Yamaguchi *et al.*, 2008). According to the previously reported results of live influenza virus particles, the influenza virion is pleiomorphic with a lipid envelope that can manifest in spherical or filamentous form. In general, the morphology of the virus is spherical or ovoid, with a diameter between 50 and 120 nm. In contrast, there is currently no report regarding images of virus particles contained in a vaccine suspension.

Fig. 2 shows TEM images of the estimated inactivated split virus particles from the vaccine solution stained with uranyl acetate. The majority of the particles still maintained their shapes similar to live viral ones. Nermut (1982) and other researchers previously reported that staining with

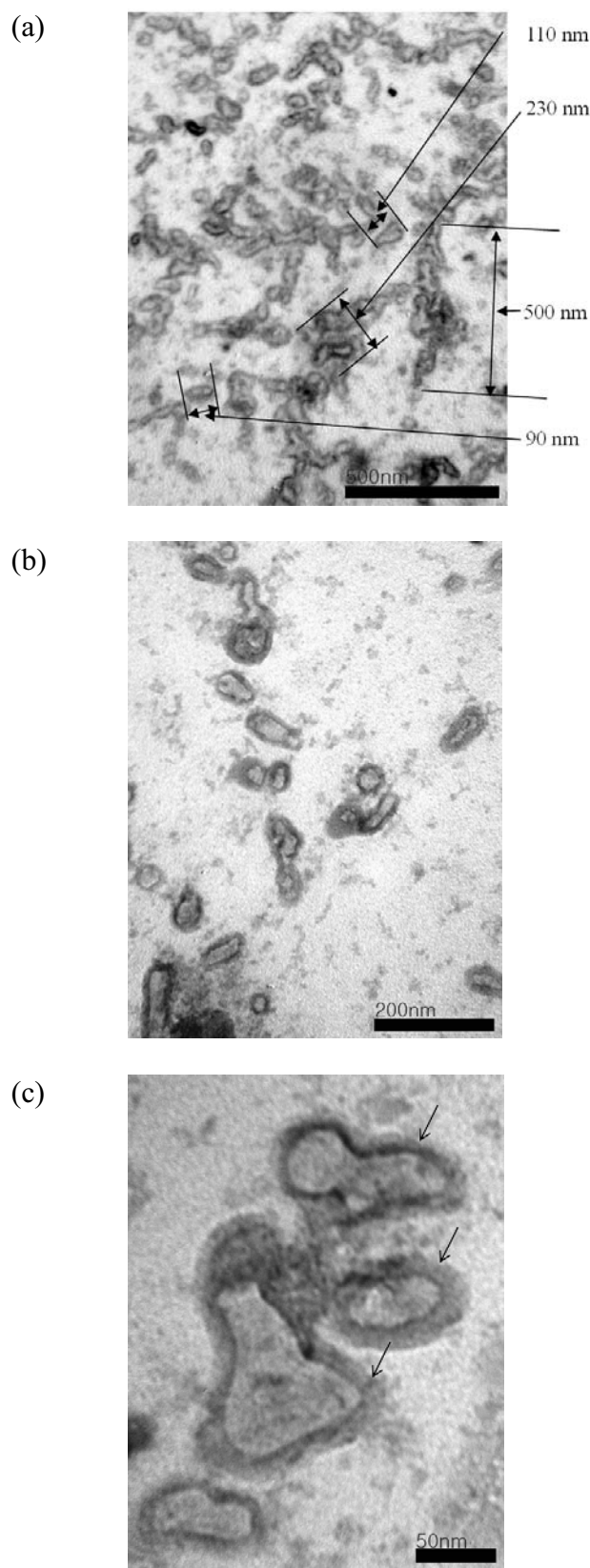


Fig. 2. Transmission electron microscopic (TEM) images of influenza A H1N1 virus vaccine particles (staining with 0.2 % uranyl acetate). The scale bars indicate (a) 500 nm, (b) 200 nm, and (c) 50 nm. Arrows in (c) indicate membrane-like structures.

uranyl acetate is superior for detection of membranous components (i.e. phospholipids, sterols, and proteins) as well as of glycoproteins associated with enveloped viruses. Therefore, as shown in Fig. 2, the dark outer shapes were membrane-like structures, based on partial selective staining with uranyl acetate. The TEM images suggest that the inactivated virus particles present in the vaccine solution maintained a very similar appearance to that of the untreated virus particles in terms of shape and membrane-like exterior structures. The calculated sizes of the particles from the image, including agglomerated particles, ranged from 60 nm to 500 nm. The primary particle (basic building particle for an aggregate) size of the agglomerates ranged from 60 nm to 120 nm. It is worth noting that the primary particles shown in Fig. 2 featured membrane-like structures on their outer surfaces, indicating that the inactivated virus particles in the vaccine solution had maintained their envelope structures. These data are valuable since this is the first report on the characterization of inactivated influenza vaccine particles by TEM.

Genetic Detection of Inactivated Influenza Viral Particles in Aerosols

Since the vaccine particles may be decomposed and lose their integrity during the aerosolization process, we adopted DNA amplifying technique to detect the presence of the vaccine particles in aerosols. Fig. 3 shows the results of the genetic detection experiments, which was newly adopted to detect the presence of even a very small amount of inactivated vaccine particles in aerosols using DNA amplification technique. An influenza virus particle contains segmented viral RNA genomes and several viral specific proteins. To confirm the actual presence of virus particles in the collected aerosols as well as in the initial vaccine solution, we chose to detect viral genomic RNA before and after the nebulization process based on the fact that nucleic acids have more advantages compared to proteins in detection - even a small amount of RNA can be easily detected after PCR amplification. For the genetic detection of inactivated viral particles in the generated aerosols, we first converted the purified the genomic RNAs to complementary DNAs (cDNAs) via reverse transcription reaction. Then, the cDNAs were PCR amplified using two primers specific to the H1 gene, and separated on an agarose gel. One fragment longer than 100 bp was

successfully amplified not only from the cDNAs of the initial vaccine solution but also from those of the collected aerosols, whereas no fragments appeared in the negative control lanes, as shown in Fig. 3. For further analysis, the amplified fragment was cloned, sequenced, and confirmed to be a perfect match with the H1 sequence of the influenza strain. These results indicate not only the fact that viral RNA can be amplified and detected from inactivated split vaccine particles, but also that the genes of virus particles were actually present in the solution before and after the nebulization process.

Size Distributions of Aerosol Particles Generated from the Inactivated Virus Vaccine Solution

Fig. 4 shows the particle size distributions of the generated aerosol particles. We used two types of aerosol measurement equipment (PSD and SMPS), each of which covers different particle size ranges; however, we could not detect any significant number of particles via PSD measurement in its effective measurement size ranges (0.5 μm to 100 μm). Therefore, we have presented the SMPS measurement results in Fig. 4 using different suspensions of varying dilution ratios. The negative control experiment using solution D revealed a significantly low aerosol particle concentration, thus confirming that the filtered and purified sterile water used for the dilution of the vaccine suspension will not significantly affect the results of aerosol particle size distribution of the vaccine particles.

The graphs in Fig. 4 show that the majority of the detected aerosol particles fell within a size range between 80 and 400 nm with a mean diameter of 130 nm. The aerosols with 80 to 120 nm diameters might include singly separated primary attenuated virus particles, based on the TEM images shown in Fig. 2. To identify some aerosol particles with diameters of 300 nm or more, we obtained scanning electron microscopic (SEM) images of aerosols sampled with a membrane filter (Nucleopore track-etched polycarbonate membrane filter, Whatman, Brentford, UK; a pore size of 0.05 μm). Relatively large salt particles with typical simple crystal structures were observed as shown in Fig. 5. Since the various salts are contained in the buffer solution of the vaccine (pH 7.2, 800 mg of NaCl, 20 mg of KCl, 115 mg of Na_2HPO_4 , 20 mg of KH_2PO_4 in 100 mL solution) to solubilize inactivated virus particles of the vaccine, salt crystals may form upon drying. Therefore, it

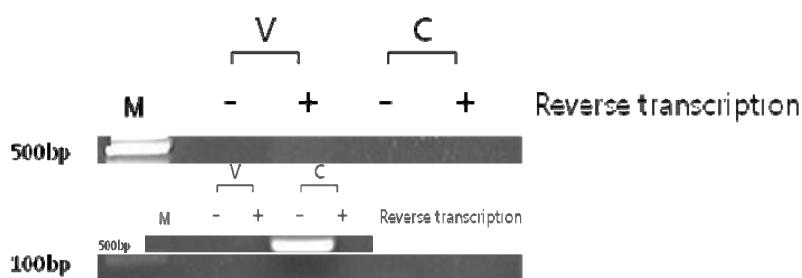


Fig. 3. Detection of viral genomic RNA in the initial vaccine solution and the collected aerosols. Viral RNAs were reverse-transcribed and PCR-amplified from the two solutions (V, the initial inactivated virus vaccine solution; C, the collected aerosols: -, the samples without reverse transcription).

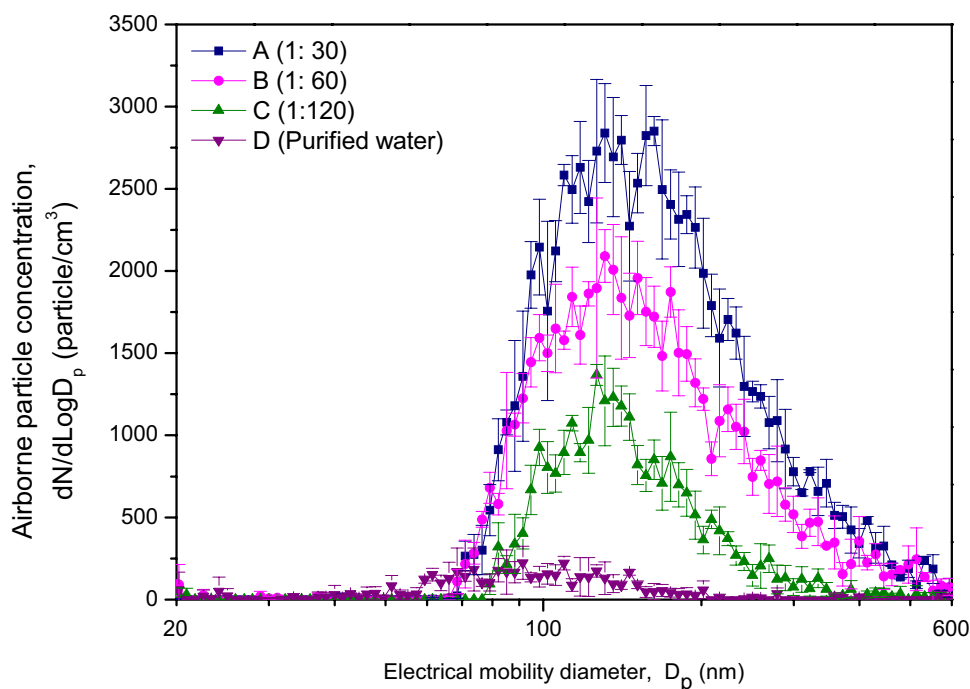


Fig. 4. Aerosol particle size distribution of influenza A H1N1 virus vaccine particles mixed with filtered sterile water generated via nebulization. Four types of suspensions with varying dilution ratios were tested: the suspension A (dilution ratio 1:30), the suspension B (dilution ratio 1:60), the suspension C (dilution ratio 1:120), and D (filtered purified sterile water for control test).

is highly possible that large size particles in the particle size distribution graphs shown in Fig. 4 are the large salt particles mixed with large agglomerated virus particles, whereas the small size particles in the distribution are singly separated attenuated virus particles mixed with tiny salt particles. Additionally, the overall shape of the particle size distributions and the mode diameter did not change with differing dilution ratios of the suspensions, as shown in Fig. 4. This observation implies that the concentration of water-soluble particles in the aerosol particles was insignificant as to affecting the sizes of the particles whereas the amount of solid (water-insoluble) components was significant in the aerosol particles. Otherwise, if the water-soluble component dominated, then the particle sizes should have changed significantly with changing dilution ratios; for example: the aerosol particles observed with suspension A should have been larger than with suspension C (we used the same nebulizer under the same flow rate conditions, and thus the liquid droplets generated were the same, except for the concentration of particles including salts contained in the droplets). Therefore, we can infer that the solid (water-insoluble) components, including virus agglomerated particles, play a dominant role in the aerosol particles when the vaccine solutions are aerosolized.

In the above experiments, however, we could not classify the vaccine aerosol particles into several size groups to investigate the characteristics of individual size groups. Separation based on aerosol particle size and the subsequent characterization of each size group is currently under investigation.

The gravitational sedimentation time of aerosolized 130

nm particles is around 340 hours for a 1.5 m falling height without the effect of convective airflow (Hinds, 1999), indicating that gravity does not affect the particles in a practical sense. The 130 nm vaccine particles are mostly affected by diffusion and convective airflow. The thermal diffusion velocity of the 130 nm aerosol particles is around ~ 0.1 m/s in all possible directions (Hinds, 1999). As the diffusion phenomena involve the movement of particles from a high concentration region to low concentration region, we can expect that 130 nm airborne vaccine particles will spread at a velocity of around 0.1 m/s toward surrounding environments via diffusion only. However, air-conditioning systems for the heating or cooling of indoor air environments are common in public facilities such as airports, hospitals, trains, and subway stations. Based on our measurement data, the air wind velocity in a typical air-conditioned indoor environment ranges from 1 m/s to 10 m/s, which will highly enhance the spread of vaccine particles. While several factors still must be considered when attempting large-scale mass vaccinations using aerosolized vaccine particles, such as side effects in immuno-compromised individuals and other ethical issues, the result of our experiment reveal that the methods described herein have some potential. Nanoparticles such as 80 nm aerosol particles are known to penetrate blood vessels in the lung and spread throughout the human body (Oberdorster *et al.*, 2007). We are currently testing the efficacy of the intranasal delivery of aerosolized vaccines for inducing a mucosal immune response in mice, as well as the effects of various vaccine particle sizes on the immune response.

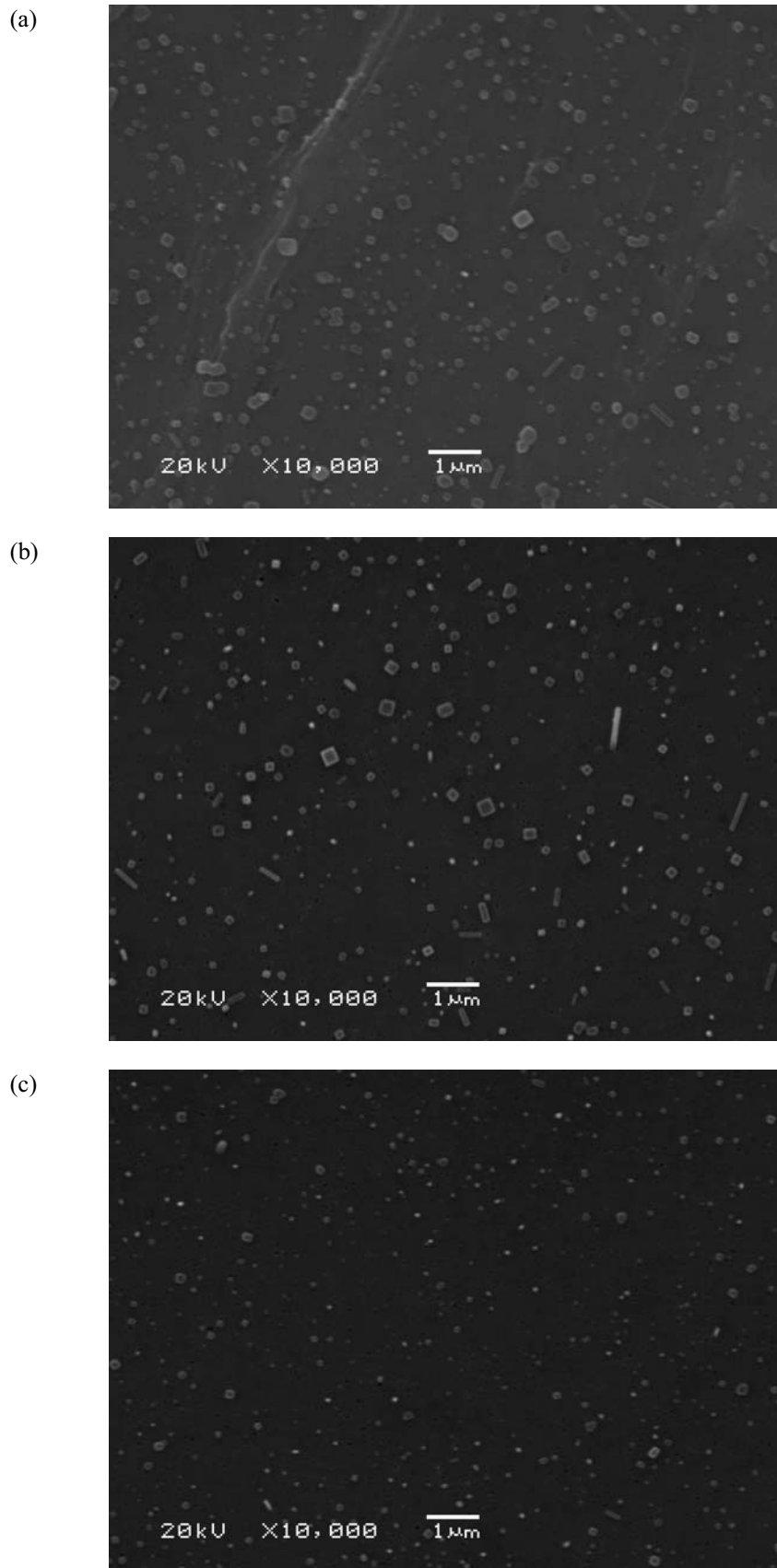


Fig. 5. Scanning electron microscopic (SEM) images of aerosolized vaccine particles sampled using a membrane filter. Aerosol particles from (a) the suspension A (dilution ratio 1:30), (b) the suspension B (dilution ratio 1:60), and (c) the suspension C (dilution ratio 1:120).

CONCLUSION

In this study, we generated aerosols with a conventional vaccine solution containing inactivated split influenza virus, and analyzed the characteristics of the aerosol particles. Clear TEM images of the virus particles in the vaccine were taken, indicating that attenuated virus particles in the vaccine actually maintained their genetic and physical viral characteristics. Therefore, vaccine solutions can be suggested as relatively safe surrogates of virulent pathogenic viruses in research. One interesting finding of this study was that genomic RNA from chemically-inactivated virus particles in a vaccine can be easily detected by simple RT-PCR methods when amplified. In addition, aerosolized vaccine particles containing inactivated split influenza viruses showed a mode diameter of 130 nm, which allows for easy entry into the human respiratory tract. Overall, the current experimental results provide information that may support vaccination using aerosolized vaccine particles for replacing painful and time-consuming injection methods.

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