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Aerosol Disinfection Capacity of Slightly Acidic Hypochlorous Acid Water Towards Newcastle Disease Virus in the Air: An *In Vivo* Experiment

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SUMMARY. Existence of bioaerosol contaminants in farms and outbreaks of some infectious organisms with the ability of transmission by air increase the need for enhancement of biosecurity, especially for the application of aerosol disinfectants. Here we selected slightly acidic hypochlorous acid water (SAHW) as a candidate and evaluated its virucidal efficacy toward a virus in the air. Three-day-old conventional chicks were challenged with 25 doses of Newcastle disease live vaccine (B1 strain) by spray with nebulizer (particle size <3 μm in diameter), while at the same time reverse osmosis water as the control and SAHW containing 50 or 100 parts per million (ppm) free available chlorine in pH 6 were sprayed on the treated chicks with other nebulizers. Exposed chicks were kept in separated cages in an isolator and observed for clinical signs. Oropharyngeal swab samples were collected from 2 to 5 days postexposure from each chick, and then the samples were titrated with primary chicken kidney cells to detect the virus. Cytopathic effects were observed, and a hemagglutination test was performed to confirm the result at 5 days postinoculation. Clinical signs (sneezing) were recorded, and the virus was isolated from the control and 50 ppm treatment groups, while no clinical signs were observed in and no virus was isolated from the 100 ppm treatment group. The virulent Newcastle disease virus (NDV) strain Sato, too, was immediately inactivated by SAHW containing 50 ppm chlorine in the aqueous phase. These data suggest that SAHW containing 100 ppm chlorine can be used for aerosol disinfection of NDV in farms.

RESUMEN. Capacidad de desinfección del agua ligeramente acidificada con ácido hipocloroso contra el virus de la enfermedad de Newcastle en el aire: Un experimento *in vivo*.

La existencia de contaminantes en forma de bioaerosol en las granjas, así como los brotes de algunos organismos infecciosos con capacidad de transmisión por el aire confirman la necesidad de mejorar la bioseguridad, especialmente mediante la aplicación de desinfectantes en aerosol. En este estudio, se seleccionó agua ligeramente acidificada con ácido hipocloroso (con las siglas en inglés: SAHW) como candidato y se evaluó su eficacia virucida hacia un virus presente en el aire. Se desafiaron pollos de tres días de edad con 25 dosis de vacuna viva contra el virus de la enfermedad de Newcastle (cepa B1) por aerosol con un nebulizador (tamaño de partícula menor de 3 μm de diámetro), mientras que al mismo tiempo se nebulizó agua producida por ósmosis inversa como control y SAHW que contenía 50 o 100 ppm de cloro libre disponible con un pH 6 sobre los pollos tratados con otros nebulizadores. Los pollos expuestos fueron mantenidos en jaulas separadas en un aislador y se observaron para detectar signos clínicos. Se recolectaron muestras de hisopos orofaríngeos de dos a cinco días después de la exposición de cada pollo, y luego las muestras se titularon en cultivos primarios de células de riñón de pollo para detectar al virus. Se observó efecto citopático, y se realizó una prueba de hemaglutinación para confirmar el resultado a los cinco días después de la inoculación. Se registraron signos clínicos (estornudos), y el virus se aisló de los grupos de control y del grupo tratado con 50 ppm, mientras que no se observaron signos clínicos y tampoco se aisló virus del grupo tratado con 100 ppm. El virus de la enfermedad de Newcastle (NDV) virulento cepa Sato, también, se inactivó inmediatamente por SAHW que contenía 50 ppm de cloro en fase acuosa. Estos datos sugieren que el SAHW que contiene 100 ppm de cloro puede ser utilizada para la desinfección por aerosol contra el virus de la enfermedad de Newcastle en las granjas.

Key words: aerosol disinfectant, biosecurity, Newcastle disease virus, spray, virus inactivation

Abbreviations: AI = avian influenza; CK = chicken kidney; DPE = days postexposure; EDTA = ethylenediaminetetraacetic acid; FBS = fetal bovine serum; MEM = minimum essential medium; MM = maintenance medium; ND = Newcastle disease; NDV= Newcastle disease virus; PBS = phosphate-buffered saline; ppm = parts per million; RO = reverse osmoses; SAHW = slightly acidic hypochlorous acid water; $TCID_{50}$ = 50% tissue culture infective dose; VN = virus neutralization

Outbreaks and fast transmission of some avian viral diseases like Newcastle disease (ND), avian influenza (AI), and infectious bronchitis, with their high morbidity and mortality rates, are largely attributed to infection via aerosol (9,12,15,22,29,31,36). High amounts of airborne pathogens are present in poultry farms. They reduce the productive capacity of the poultry and act as a potential threat for the poultry industry, as well as for the farm personnel (6,11,14,38,40). Infected birds shed viruses directly to the air by droplets during sneezing or coughing and indirectly through feces (4,28,30), thus contaminating the air and floor of the farms and the objects that are nearby. ND virus (NDV) remains infective for a long time in the environment, on the surfaces of contaminated objects and in the air (4,16,20,33). Susceptible hosts contract the virus directly via inhalation of contaminated air or indirectly through ingestion of contaminated materials (1,22). Among the avian diseases, ND is one of the most fatal, with a large numbers of outbreaks and host species (2,3,18,28). The stability and transmissibility of aerosolized NDV has been tested under different conditions (20,22,33). Since the virus is inhaled directly into the

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deeper respiratory system, smaller amounts of the virus are required to infect the chicks (7,8,21). Current strategies to control ND, AI, or other highly contagious poultry diseases include vaccinations, surveillance, quarantine, depopulation, disposal, and decontamination of the poultry farms, which are very cost effective (3,4,5,10). However, these strategies are not adequately successful, and ND remains a potential threat for poultry producers worldwide. Enhancement of the biosecurity in farms is the most effective way to control avian diseases. As biosecurity stands for measures conducted in order to keep infectious agents away from farms and to limit the chance of their transmission and outbreaks, one of those measures that effectively can help the farmers to enhance their farm's biosecurity is implementation of a perfect spray system with application of an ideal aerosol disinfectant. Since slightly acidic hypochlorous acid water (SAHW) has very fast and strong efficacy against pathogens (13), we evaluated its efficacy toward NDV in the air to facilitate bioaerosol decontamination as an *in vivo* experiment.

MATERIALS AND METHODS

Slightly acidic hypochlorous acid water. Slightly acidic hypochlorous acid water containing free chlorine at the rate of 50 parts per million (ppm) was prepared by a generator "Well Clean-TE" (OSG Co., Ltd., Osaka, Japan) in our laboratory with normal tap water on the day of use. SAHW containing 100 ppm chlorine was kindly supplied by OSG Co., Ltd.

Aerosol sprayer and spray boxes. Nebulizers (NE-C28 Camp A-I-R) with the ability to produce small aerosol particles (size <3 μm in diameter) were purchased from Omron Corp. (Kyoto, Japan). Plastic boxes measuring 513 × 359 × 230 mm in size were purchased from a local market.

Animal. Animal work was performed in strict accordance with Animal Care guidelines of Tokyo University of Agriculture and Technology (Tokyo, Japan) with permit numbers 25–37 and 26–45. Day-old commercial chicks, with no vaccination, hereafter designated "conventional chicks," were purchased from Kanto Co., Ltd. (Gunma, Japan), labeled, and settled in rat cages (CLEA-0108-3, Clea Japan, Inc., Tokyo, Japan) inside the isolator (CL-5443, Clea Japan) and kept until 3 days old, then used for the experiments.

Virus and cell. ND live vaccine (NDV-B1: lyophilized, 5000 doses, $>10^{9.2}$ 50% egg infected dose per vial) was purchased from Nisseiken Co., Ltd. (Tokyo, Japan). On the day of use, the ND vaccine was reconstituted in 50 ml of double distilled water. After its titration on the chicken kidney (CK) cells, the titer was 7.25 $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$)/ml, and we confirmed the 100 doses/ml. Virulent NDV strain Sato (32) also was used. The virus was propagated in 10-day-old embryonated eggs, and the amnio-allantoic fluids were harvested, aliquotted, and stored at −80 C. Primary CK cells were prepared from kidneys of 1- to 7-day-old chicks as described (19,34). Briefly, chicks were dissected, and their kidneys were removed using aseptic technique then washed with phosphate-buffered saline (PBS: 0.14 M NaCl, 2 mM KCl, 3 mM Na_2HPO_4, 1.5 mKH_2PO_4, pH 7.4). After stirring with a magnetic bar for 5 min, tissues were treated sequentially by three to four times trypsinization for 5 min with trypsin ethylenediaminetetraacetic acid (EDTA; 0.05% trypsin, 0.05 mM EDTA, 1× PBS). The resulting cell suspension was centrifuged at 440 × *g* for 5 min. The cell pellet was resuspended in growth medium shown below and cultured in plates at 0.3% cell concentration. The cell suspension was seeded at 4 ml onto tissue culture dishes of 60 mm in diameter and 100 μl per well, over 96 microplates. The growth medium consisted of Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.3% tryptose phosphate broth, penicillin 100 IU/ml, streptomycin 100 μg/ml, Amphotericin B 0.5 μg/ml, 4 mM L-glutamine, and 5% fetal bovine serum (FBS).

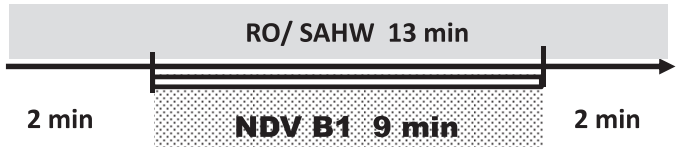

Fig. 1. A scheme of the spraying duration of ND vaccine virus and SAHW/RO. SAHW or RO water were sprayed for 13 min (2 min before and 2 min after NDV spraying). Within 9 min, 25 doses of ND vaccine were sprayed.

***In vitro* experiment using virulent strain Sato.** To confirm the virucidal efficacy of SAHW against the virulent strain Sato, 225 μl of SAHW containing 50 ppm chlorine was mixed with 50 μl of NDV strain Sato and kept for 5 sec. Then 225 μl of FBS was added to stop the activity of SAHW. The remaining virus was titrated on CK cells in a 96 microplate.

Sampling procedure and virus isolation. Oropharyngeal swabs were collected using a Rayon cotton bulb swab from Eiken Chemical Co., Ltd. (Tochigi, Japan), from 2–5 days postexposure (DPE) to ND live vaccine, from all chicks. The swabs were put in vials containing transport medium (brain heart infusion broth 3.7%, penicillin 1000 IU/ml, streptomycin 1 mg/ml, Amphotericin B 5 μg/ml) (26), vortexed, and kept for 1 hr at 4 C, then stored at −30 C up to the day of inoculation. Swab samples were titrated on a monolayer of CK cells in 96-well microplates. Serial tenfold dilution was prepared per swab sample in maintenance medium (MM) shown below and inoculated to CK cells seeded in 96-well plates of 100 μl/well and four wells per dilution. The MM was prepared from Eagle's MEM supplemented with tryptose phosphate broth 0.3%, penicillin 100 IU/ml, streptomycin 100 μg/ml, Amphotericin B 0.5 μg/ml, and 4 mM L-glutamine. Cytopathic effects were observed daily in the inoculated plate, and hemagglutination test was performed at 5 days postinoculation to confirm the result.

Virus neutralization (VN) test. The VN test was performed in CK cells to calculate the chicken's maternal antibody titer using the plaque-reduction method, with a constant amount of virus and varying serum dilution as described (27). Briefly, sera samples were collected from conventional chicks before virus spraying (at 3 days old), diluted in a serial fourfold dilution in PBS, and mixed with the equal volume of NDV strain Sato (32). The neutralizing antibody titer was calculated at 50% plaque-reduction point by Behrens-Kärber's method (24).

Experimental design. *Experiment 1: Aerosol delivery of vaccine virus and preventive effects of maternal immunity.* The experiment was designed to find the chicken's maternal immunity titer, and the required dose of vaccine was designed to infect chicks to the maximum (100%). Three-day-old conventional chicks were numbered, and 0.5 ml blood was collected from each chick to analyze the maternal antibody titer. Then, the chicks were divided into four groups of five birds and placed in the spray boxes separately. Different doses of NDV B1 (10, 25, 50, and 100 doses/2 ml) were prepared from the vaccine solution (100 doses/ml) with PBS, and the 2 ml of volume was sprayed completely into each box within 9 min by nebulizer (Fig. 1). The lids of the boxes were kept closed for 5 min after spraying to let chicks inhale the virus (Fig. 2). Then, the chicks were transferred to their cages in a single isolator. Chicks were observed for clinical signs, and oropharyngeal swab samples were collected from 1 to 6 DPE per chick into viral transport medium to isolate the virus, followed by chick dissection at 6 DPE. Virus neutralization assay was performed to find their maternal antibody titer. This experiment was repeated two times, except for the group exposed to 100 doses.

Experiment 2: Aerosol disinfection capacity in the air. This experiment was designed to evaluate virucidal capacity of SAHW toward NDV in the air. Fifteen 3-day-old conventional chicks were numbered and divided into three groups with five birds per group. In Experiment 1, we determined the NDV vaccine dose that showed 100% infection and then used that dose to separately spray each group in Experiment 2 (Fig. 2). The control group was sprayed with reverse osmosis (RO) water, and SAHW treatment groups were sprayed with SAHW

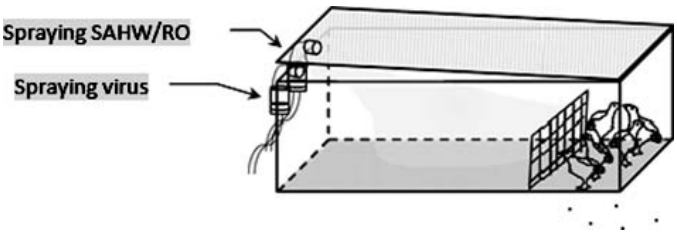

Fig. 2. Spraying by nebulizers of ND vaccine virus and SAHW. Chicks were kept in one side of the box with wire net, while ND vaccine and SAHW/RO were sprayed on the other side.

containing 50 ppm or 100 ppm of chlorine. SAHW or RO water was sprayed for 13 min; from 2 min before to 2 min after the NDV spraying, which was conducted, thus, for 9 min (Fig. 1). Chicks were kept in the box with the lid closed for 5 min to let them inhale the remaining virus then transferred to their cages in the isolator. Clinical signs were observed, and sampling was performed from 2 to 5 DPE, as in Experiment 1. Chicks were dissected at 5 DPE after sampling. This experiment was repeated three times, except for the 50 ppm treatment (repeated twice).

Experiment 3: Influence of SAHW on normal growth. This experiment was designed to learn whether SAHW affects the chick's normal growth or causes any abnormal conditions. Fifteen 3-day-old conventional chicks were divided into three groups of five birds, one group for the control and two for the treatments. For treatment 1, chicks were weighed and placed in the spray box/group, then 100 ppm SAHW was sprayed for 6 days, daily for 13 min, twice: once in the morning and once in the afternoon; whereas for treatment 2, 100 ppm SAHW spraying was conducted once a day, every morning, for a total of 6 days. The control group received RO water spray for 6 days, twice a day, as in Treatment 1. Chicks were observed for any abnormal condition during the experiment. After 6 days of SAHW spraying, chicks were weighed, and their body weight was statistically analyzed using Prism v.6.05 (Trail) software (GraphPad Software Inc. La Jolla, CA), and values are expressed as mean ± SD. One-way ANOVA followed by Bonferroni's multiple comparisons test was used to analyze and compare body weights between the control and treatments. This experiment was done one time.

RESULTS

***In vitro* experiment using the virulent strain Sato.** In the aqueous phase experiment, the virulent strain Sato was inactivated down to the level of detection limit (2.5 $TCID_{50}$/ml) within 5 sec (data not shown).

Experiment 1: Showing 100% chicken infection and their maternal immunity titer. Most of the vaccine-sprayed chicks started virus shedding at 2 DPE and continued up to 5 DPE, while some chicks started shedding from 1 DPE and some continued up to 6 DPE (Table 1). In the first group, which received 10 doses of the vaccine, chicks number 2 and 4 were infected, while other chicks were not, and chick number 5 was found infected at 6 DPE, probably due to contact with the infected birds in the same cage. However, in groups 2–4, which received higher doses of the vaccine virus (25–100 doses), all chicks were infected, and the result from the VN test showed that all of them had high titers of maternal immunity (Table 1).

Experiment 2: Showing the ability of SAHW to inactivate NDV in the air. In the control group receiving the vaccine virus at 25 doses sprayed with RO water, all chicks showed sneezing as a clinical sign from 3 DPE, and the virus was recovered from their oropharyngeal swab samples from 2 DPE (Table 2). In the 50 ppm SAHW treatment group, all chicks showed sneezing, and the virus recovered from their oropharyngeal swab samples too, while in the 100 ppm SAHW treatment group receiving ND vaccine, no clinical sign was observed, and no virus was isolated from their oropharyngeal swab samples (Table 2), except for one chick, which was found infected on 5 DPE. There was no significant difference in their gained weight within 5 days of the experiment (data not shown).

Table 1. Determination of Newcastle disease vaccine dose for 100% infectivity.[A]

Vaccine doses	Chick No.	Days postexposure[B] 1	2	3	4	5	6	VN titer[C]
10	1	−	−	−	−	−	−	956.7
	2	−	−	+	+	+	+	347.7
	3	−	−	−	−	−	−	1054.0
	4	−	+	+	+	−	−	249.3
	5	−	−	−	−	−	+	>1280.0
25	6	−	+	+	+	+	+	766.3
	7	−	+	+	+	+	+	1025.0
	8	−	+	+	+	+	+	>1280.0
	9	−	+	+	+	+	−	>1280.0
	10	+	+	+	+	+	+	290.4
50	11	−	+	+	+	+	−	844.4
	12	+	+	+	+	+	+	357.5
	13	−	+	+	+	+	−	1068.0
	14	−	+	+	+	−	−	>1280.0
	15	−	−	+	+	+	+	1178.0
100	16	−	+	+	+	+	+	>605.4
	17	+	+	+	+	+	+	1280
	18	−	+	+	+	+	−	320.0
	19	−	+	+	+	+	−	>1211
	20	+	+	+	+	+	+	>1178

[A]The preliminary experiment was conducted to find the required doses of NDV, which are sufficient to infect all chicks in a group having maternal immunity upon the day of vaccination, at the age of 3 days. Different doses of NDV-B1 (10, 25, 50, or 100 doses per box) were sprayed by nebulizer. VN titers were shown at 3 days old.

[B]Plus sign indicates virus was isolated from an oropharyngeal swab. Minus sign indicates virus was not isolated from an oropharyngeal swab.

[C]VN = virus neutralization.

Table 2. The virucidal efficacy of SAHW towards Newcastle disease virus in the air.[A]

Groups	Challenge No.	Days postexposure			
		2	3	4	5
0 ppm	1	5/5[B]	5/5	5/5	5/5
	2	4/5	5/5	5/5	5/5
	3	4/5	5/5	5/5	4/5
50 ppm	1	2/5	5/5	5/5	4/5
	2	3/5	5/5	5/5	5/5
	3	NC[C]	NC	NC	NC
100 ppm	1	0/5	0/5	0/5	1/5
	2	0/5	0/5	0/5	0/5
	3	0/5	0/5	0/5	0/5

[A]The vaccine virus at 25 doses was sprayed with RO water (0 ppm) or with SAHW (50 or 100 ppm). The experiments were repeated three times.
[B]Asterisk indicates number of infected chicks/challenged chicks.
[C]NC = not challenged.

Experiment 3: Monitoring effects of SAHW on the normal growth of chicks. The chicks that were sprayed with SAHW at 100 ppm free available chlorine for 13 min once or twice per day gained body weight not significantly in difference from the control group, which was sprayed with RO water twice a day for 13 min for a total of 6 days (Table 3). There were no abnormal conditions observed in any groups of chicks.

DISCUSSION

Biosecurity programs have a critical role in the control of all avian infectious diseases. Establishment of an effective spray system with application of a broad spectrum aerosol disinfectant, without causing harmful effects to the poultry, is very vital for infectious disease prevention and control. The essential way to control and prevent those diseases that are airborne in the poultry industry is inactivation of infectious agents by spraying disinfectants in the air. It limits the chance of their transmission and outbreaks within the farms or to other farms and flocks.

Chlorine compounds are very popular for their broad and strong disinfection ability, and SAHW, which is one of the chlorine-containing solutions, showed a very fast and strong efficacy against AI virus within a short contact time (5 sec), *in vitro* (13). In the present experiment, the virulent NDV strain Sato was also inactivated within 5 sec in the aqueous phase. Our findings from the present study confirm, thus, remarkable aerosol disinfection ability against NDV in the air.

In Experiment 1, at 1 DPE, the virus was not detected in most of the collected samples (Table 1). This means that the sprayed viruses at 10–100 doses were inadequate to be detected directly on CK cells, or that the virus was in the eclipse period in the chicks. In our preliminary experiments, the sprayed virus in the boxes could not be recovered in our systems using the Rayon sheets or dishes. With the *in vivo* system using chicks, the sprayed virus was detected as shown in Tables 1 and 2. This *in vivo* system seems to be very sensitive to detect the virus in the air. In the box, the sprayed virus reaches the chicks within 5 sec, thus exhibiting immediate virucidal efficacy required to stop the infection.

Our findings also show that chicks can be infected in spite of the presence of high maternal antibody titer and can shed a high amount of virus to the environment following infection. The findings are compatible with other studies and suggest that spraying the vaccine virus with high doses allows the vaccine virus to infect chicks even though the chicks have high maternal antibodies (10,35). According to findings by Hugh-Jones *et al.* (16) and May *et al.* (25), NDV could be found for up to 1 hr in the open air with variable humidity and wind conditions, which increases the chance for transmission and outbreaks. Presence of viruses in the air of farms and their circulation within the poultry are very critical for some diseases, like AI, with the ability to mutate and possibly bring about the emergence of a highly pathogenic strain (17,23,37).

Spraying SAHW 100 ppm was able to inactivate NDV in the air, except one chick, while its 50 ppm variant lost its efficacy after spraying, probably due to free chlorine loss during travel of the aerosolized solution over a long distance (13,39).

Within the safety test that we performed in the present study, there were no observable abnormal conditions in chicks nor difference in their gained weight (Table 3), which means that SAHW is safe and has no harmful effects on the animals present at farms. Application of SAHW in adequate concentration, from an appropriate distance by a spraying system with the ability of producing aerosol particles inside populated poultry farms, will potentially reduce the chance for aerogenic infection causing outbreaks and will limit virus transmission from one site to another. Lower cost, broad spectrum, easy mass administration, availability, and safety are the most important factors that may encourage farmers to apply this product in their animal farms.

Table 3. SAHW's (100 ppm) effects on the normal growth within the study period.[A]

Groups treated by	Chick's body weight (g)		Gained weight (g)	
	At 3 days	At 9 days	Per 6 days	Per 1 day
RO water twice	44.9 ± 2.46	91.5 ± 3.38	46.6 ± 1.87	7.77 ± 0.31
SAHW once	46.7 ± 1.52	94.6 ± 2.48	47.8 ± 1.39	7.98 ± 0.24
SAHW twice	44.9 ± 1.08	91.7 ± 2.06	46.7 ± 1.44	7.79 ± 0.23

[A]Control group was sprayed with RO water twice a day, while treated groups were sprayed with SAHW once or twice a day (every time for 13 min), for a total of 6 days. Data represent chicks body weight (mean ± SD), and statistically there was no significant difference ($P > 0.05$).

All in all, our results confirm the fast and efficient aerosol disinfection capacity of SAHW against NDV in the air and show that this substance can indeed protect chickens from infection. In addition, the results suggest application of the higher concentration of SAHW we used, with a perfect spray system, from an appropriate distance, can lead to an immediate effect. Since this study was performed under laboratory conditions, further investigation may be needed to approve its application at farms.

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