FULL PAPER *Avian Pathology*

Evaluation of sprayed hypochlorous acid solutions for their virucidal activity against avian influenza virus through *in vitro* experiments

Hakimullah HAKIM[1,2)], Chanathip THAMMAKARN[1,2)], Atsushi SUGURO[1)], Yuki ISHIDA[1)], Akinobu KAWAMURA[1)], Miho TAMURA[1)], Keisuke SATOH[1)], Misato TSUJIMURA[1)], Tomomi HASEGAWA[1)] and Kazuaki TAKEHARA[1,2)]*

[1)]*Laboratory of Animal Health, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3–5–8, Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan*
[2)]*The United Graduate School of Veterinary Science, Gifu University, 1–1, Yanagido, Gifu 501-1193, Japan*

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ABSTRACT. Hypochlorous acid (HOCl) solutions were evaluated for their virucidal ability against a low pathogenic avian influenza virus (AIV), H7N1. HOCl solutions containing 50, 100 and 200 ppm chlorine (pH 6) or their sprayed solutions (harvested in dishes placed at 1 or 30 cm distance between the spray nozzle and dish) were mixed with the virus with or without organic materials (5% fetal bovine serum: FBS). Under plain diluent conditions (without FBS), harvested solutions of HOCl after spraying could decrease the AIV titer by more than 1,000 times, to an undetectable level (< 2.5 log10$TCID_{50}$/m*l*) within 5 sec, with the exception of the 50 ppm solution harvested after spraying at the distance of 30 cm. Under the dirty conditions (in the presence of 5% FBS), they lost their virucidal activity. When HOCl solutions were sprayed directly on the virus on rayon sheets for 10 sec, the solutions of 100 and 200 ppm could inactivate AIV immediately after spraying, while 50 ppm solution required at least 3 min of contact time. In the indirect spray form, after 10 sec of spraying, the lids of the dishes were opened to expose the virus on rayon sheets to HOCl. In this form, the 200 ppm solution inactivated AIV within 10 min of contact, while 50 and 100 ppm could not inactivate it. These data suggest that HOCl can be used in spray form to inactivate AIV at the farm level.
KEY WORDS: aerosol disinfection, avian influenza, biosecurity, hypochlorous acid solution, spray

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Waterfowl and shorebirds harbor and share hemagglutinin (HA) (1–16) and neuraminidase (NA) (1–9) subtypes of influenza A viruses and interact in nature with a broad range of avian and mammalian species to which they may transmit such viruses [1]. Studies show that infected birds start virus shedding at the second day after infection [13, 24]. Transmission may occur via direct and indirect contact [1, 30, 31]. Avian influenza virus (AIV) has been shown to persist in an infective state for a long time at different pH levels (at pH 5, up to 18 hr, while at pH 7 to 9, more than 24 hr) [21] and on various surfaces and surface water [3, 23]. It can persist in an infective state on nonporous surfaces for days and can persist in an infective state on a porous surface, such as tissue paper, cloth and tissue, for up to 12 hr [28]; nevertheless, the lipid-enveloped structure of AIV increases its sensitivity to disinfectants, dehydration, detergents and surfactants [4, 14].

Disinfectants, such as hypochlorite, alkalis, oxidizing agents, alcohols and aldehydes, are all effective against AIV within a relatively short period of contact [15], but the presence of organic materials in the liquids or application area has been found to attenuate their disinfection ability [17, 19]. Control of avian influenza (AI) is extremely difficult due to its high level of contagiousness, so the best way to combat with this plague is to enhance biosecurity. Vaccination can be a way to prevent its outbreaks, as some countries are implementing it [2, 22], but differentiation of infected birds from vaccinated birds (DIVA), wild migratory birds and the various AIV subtypes without antigenic stability are problems for poultry immunization [6, 25, 26].

The main transmission modes of AIV are contact infection and droplet infection [1]; however, airborne transmission of AIV has also been demonstrated [30]. Inactivation of AIV on the surfaces of objects or in the air at poultry farms would significantly reduce and or limit the chance for its circulation and outbreaks. Many disinfectants have been evaluated for their inactivation ability, but there is still a need for their evaluation under different conditions and in different ways. Discovery of an effective aerosol disinfectant with applicability at farms that raise animals is a very important need to reduce the *bioaerosol* contaminants on poultry farms, and it would help farmers to take a meaningful step towards disease prevention and control [9]. As application of disinfectants in the presence of these kinds of animals, whose products are used for foods, requires high safety for the animals and their products, chlorine-based compounds appear to be the best choice for the mentioned purpose, and recently, some of these compounds have been evaluated for disinfection in the food industry or on farms [8, 11]; however, there is still a need for further evaluations. Hypochlorous acid (HOCl) solution is one of the chlorine byproducts obtained by dissolving chlorine in water. It is a weak acid and forms the most active ingredient in solutions; after dissolving chlorine

*CORRESPONDENCE TO: TAKEHARA, K., Laboratory of Animal Health, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3–5–8, Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan. e-mail: takehara@cc.tuat.ac.jp

in water, it splits into two forms, HOCl and hypochlorite ion (HCl^-), and the virucidal ability of solutions containing a high amount of HOCl is better than those containing HCl^-, because the virucidal ability of HOCl is 120 times higher than that of HCl^-. Furthermore, the level of free available chlorine in chlorine-based compounds (often called HOCl) is highest in pH 5 solutions [29]. Therefore, we evaluated HOCl solutions (pH 6) for their virucidal efficacy against AIV in aqueous, direct and indirect spray forms, and the aim for the present study was to determine whether they can inactivate AIV on the surface of objects and whether they retain their efficacy after spraying. If an indirect spray (aerosol) would be effective, it would be useful for inactivation of AIV on the surfaces behind objects.

MATERIALS AND METHODS

HOCl solutions: An HOCl solution, slightly acidic hypochlorous water (SAHW) containing 50 ppm chlorine, was prepared by a "Well Clean TE" Hi-Clo Soft Acidic Water Generator (OSG Co., Ltd., Osaka, Japan) in our laboratory with normal tap water on the day of use. HOCl solutions containing 100 and 200 ppm chlorine were kindly supplied by OSG Co., Ltd.

A Nanoscale aerosol sprayer was kindly prepared by Nanoscale Co., Ltd. (Kawasaki, Japan) with the ability to spray 500 m*l*/hr with a particle size diameter of less than 20 μm. Spray boxes measuring W360×D290×H112 mm were purchased from a local market.

Virus and cells: A low pathogenic AIV (LPAIV), A/duck/Aomori/395/04 (H7N1), isolated from wild ducks [12] was propagated in embryonated chicken eggs, and infected amnio-allantoic fluid (AAF) was harvested and centrifuged at 440 × g for 15 min, aliquoted and then stored at −80°C until the day of use. The virus was titrated on Madin-Darby canine kidney (MDCK) cells in 96-well tissue culture plates (4 wells per dilution, 200 μl final volume in each well) with cell culture medium containing 1 μg/m*l* trypsin (final concentration, trypsin from bovine pancreas 10,000 BAEE units/mg protein, Sigma, St. Louis, MO, U.S.A.), and the 50% tissue culture infective dose ($TCID_{50}$)/m*l* was determined by the method of Behrens and Kärber [16].

Experimental design:

Experiment 1. reaction in liquid. Two hundred twenty-five microliters of HOCl solutions or harvested solutions after spraying on dishes placed with a distance of 1 or 30 cm between the spray nozzle and dish (Fig. 1), respectively, were mixed with 50 μl of AIV and kept like that for an exposure time of 5 sec. Then, 225 μl of fetal bovine serum (FBS) was added on them to stop the activity of the HOCl solutions. To appraise their inactivation ability in the presence of 5% FBS as a model for organic materials, a solution of 5% FBS in HOCl solution v/v was prepared (225 μl), and then, 50 μl of AIV was added to it. It was kept like that for an exposure time of 5 sec, and finally the solution's activity was stopped by adding 225 μl FBS. To determine whether addition of 225 μl of FBS can stop the activity of the same volume of solution, it was mixed with the same volume of solution, and then, 50 μl of AIV was added to it, using a vortex mixer to mix. Fifty microliters of AIV was added to 450 μl maintenance medium (MM) in a micro tube as a positive control. All the experiments were carried out in triplicate (as well as experiments 2 and 3). MM was prepared from Eagle's minimum essential medium (MEM: Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with penicillin 100 IU/m*l*, streptomycin 100 μg/m*l*, amphotericin B 0.5 μg/m*l* and 4mM L-glutamine.

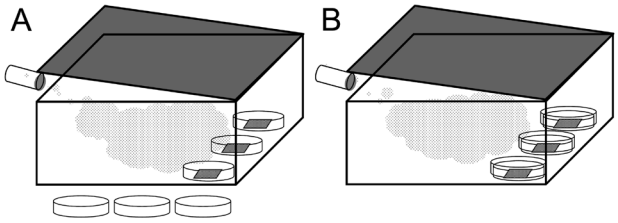

Fig. 1. Spray system. (A) Direct spray: the lid of the dish was away during solution spray. (B) Indirect spray (aerosol): the lid of the dish was closed during solution spray, after stopping spray it was removed and the dish was kept for 10 min inside the box.

Experiment 2. reaction following application of the direct spray form. One hundred microliters of AIV was inoculated onto a 3 × 3 cm^2 cut rayon sheet placed onto a 5 × 5 cm^2 glass inside a 90 mm diameter Petri dish without a lid and transferred into the spray box, and then, HOCl solutions were subsequently sprayed directly in the spray box onto the inoculated rayon sheet at a distance of 30 cm (between the spray nozzle and sample) (Fig. 1A), respectively, for a certain period of time (10 sec). Reverse osmosis (RO) water was sprayed as the positive control and was used under the same condition as the other sprays. After spraying, the samples (rayon sheet and glass) were transferred directly or after a few min of contact into stomacher bags (size 100 × 150 × 0.09 mm, capacity 80 m*l*; Organo Corporation, Tokyo, Japan) containing 900 μl MM to stop the solution activity, and the virus was extracted by mixing with a BagMixer (MiniMix 100W CC, Practical Japan Inc., Chiba, Japan), centrifuged and harvested from the supernatant as the remaining virus. Samples were prepared by serial 10-fold dilution in MM and inoculated to MDCK cells for titration.

Experiment 3. reaction in indirect spray form. Virus samples were prepared as in experiment 2 and transferred into the spray box. The lid of the Petri dish was kept closed, while HOCl solutions were sprayed into the spray box for a certain period of time (Fig. 1B). The lid of the Petri dish was opened, and the lid of the spray box was immediately closed for certain contact times to let the particles of the solution react with the virus present on the rayon sheet. RO water was sprayed on the positive control and was used under the same conditions as the other sprays. After the contact time, the same procedure was repeated as in experiment 2.

Neutralizing index (NI): A numerical method was used to express the ability of a *tpc-ta* agent to inactivate viruses as previously described [14]. An NI of virus inactivation was used to evaluate the efficacy of the agents. The NI of virus

inactivation is calculated using the following equation:

NI=tpc − ta,

where *tpc* is the titer converted into an index in log10 of the positive control, and *ta* is the converted titer of the recovered virus from the agent-treated sample. Inactivation of viruses was considered effective when NI was ≥3 [14].

RESULTS

When AIV was used without dilution, the AIV titer was decreased by more than 3 log10 $TCID_{50}$/m*l* with the 200 ppm HOCl solution, but not with the 100 or 50 ppm solution. Therefore, AIV was diluted ten times with phosphate buffered saline (PBS, pH 7.4) just before use to evaluate the virucidal ability of the HOCl solutions. As ten times diluted virus was used for this study, the virus detection limit was ≥2.5 log10 $TCID_{50}$/m*l*. At 0 sec, which is when 50% FBS was added to the solution before adding AIV to it, the titer of AIV was not reduced at all (NI=0). This means that the virucidal reaction can be stopped at any time by adding 50% FBS to the reaction tube.

Table 1 summarizes the inactivation of AIV in liquid form. With 50 ppm of HOCl solution or its harvested solution from a distance of 1 cm after spraying, the titer of AIV was reduced from $10^{7.8}$ $TCID_{50}$/m*l* to ≤ $10^{2.5}$ $TCID_{50}$/m*l* (NI ≥ 5.3) within 5 sec of contact; however, its harvested solution from a distance 30 cm after spraying reduced the titer of AIV from $10^{7.6}$ $TCID_{50}$/m*l* to $10^{6.6}$ $TCID_{50}$/m*l* (NI=1.0) and could not further reduce the titer of the virus even though with much longer incubation times (3 min). The 100 and 200 ppm HOCl solutions had high efficacy and reduced the AIV titer from $10^{7.8}$ $TCID_{50}$/m*l* to ≤ $10^{2.5}$ $TCID_{50}$/m*l* (NI ≥ 5.3) and from $10^{8.0}$ $TCID_{50}$/m*l* to ≤ $10^{2.5}$ $TCID_{50}$/m*l* (NI ≥ 5.5), respectively, after spraying from a distance of 30 cm. In reactions in the presence of 5% FBS in the solution, all solutions lost their efficacy (NI=0).

Table 2 summarizes the data for the direct spray form. Firstly, RO water was sprayed directly onto the rayon sheets or indirectly inside the spray box for different spray times, and then, the amount of RO water present on the rayon sheets was observed to determine the level of humidity; also, its weight was checked by balance to determine the amount of water. With 10 sec of direct spraying onto the sheet, around 280 μl RO water was present on the sheet, and with the same duration of indirect spraying into the box, the box was found to be full of RO water particles; therefore, a spray time of 10 sec was selected as desired spray time for evaluation of solutions.

To evaluate HOCl solutions in the different spray forms, ten times diluted AIV was inoculated to the rayon sheets, and the recovery from sheets was around $10^{5.7}$ $TCID_{50}$/m*l*. In comparison to the original ten times diluted virus titer ($10^{6.7}$ $TCID_{50}$/m*l*), about a 10 times reduction was observed.

With the spray time of 10 sec for the 50 ppm solution, the titer of AIV reduced instantly from $10^{5.5}$ $TCID_{50}$/m*l* to $10^{4.17}$ $TCID_{50}$/m*l* (NI=1.33) directly after spraying, and the virus titer reduced from $10^{5.7}$ $TCID_{50}$/m*l* to ≤ $10^{2.5}$ $TCID_{50}$/m*l* (NI ≥ 3.2) within 3 min of contact. On the other hand, the 100 and 200 ppm solutions were able to reduce the titer of AIV from $10^{5.5}$ $TCID_{50}$/m*l* to ≤ $10^{2.5}$ $TCID_{50}$/m*l* (NI ≥ 3), and $10^{5.62}$ $TCID_{50}$/m*l* to ≤ $10^{2.5}$ $TCID_{50}$/m*l* (NI ≥ 3.1), respectively, directly after spraying.

Table 3 shows the indirect spray results. By spraying the hypochlorous acid solutions inside the spray box for 10 sec and leaving it there for 10 min of contact, the 50 ppm solution reduced the titer of AIV from $10^{5.5}$ $TCID_{50}$/m*l* to $10^{4.5}$ $TCID_{50}$/m*l* (NI=1), the 100 ppm solution reduced the titer from $10^{5.96}$ $TCID_{50}$/m*l* to $10^{4.03}$ $TCID_{50}$/m*l* (NI=1.94), and the 200 ppm solution reduced the titer from $10^{5.78}$ $TCID_{50}$/m*l* to ≤ $10^{2.5}$ $TCID_{50}$/m*l* (NI ≥ 3.2), respectively.

Table 1. Inactivation of AIV in liquid with an exposure time of 5 sec by the original or harvested HOCl solutions after spraying

HOCl[a] (ppm)	Harvested after spraying/cm	Log 10 $TCID_{50}$/m*l*		
		PC[b]	RV[c]	NI[d]
50	–	7.7 ± 0.55	≤ 2.5	≥ 5.2
	1	7.8 ± 0.76	≤ 2.5	≥ 5.3
	30	7.6 ± 0.68	6.6 ± 0.72	1
100	–	NT[e]	NT	NT
	1	NT	NT	NT
	30	7.8 ± 0.55	≤ 2.5	≥ 5.3
200	–	NT	NT	NT
	1	NT	NT	NT
	30	8.0 ± 0.00	≤ 2.5	≥ 5.5

a: HOCl=hypochlorous acid, b: PC=positive control: AIV was mixed with MM, but not HOCl solutions, c: RV=remaining virus after treatment with HOCl solutions, d: NI=neutralization index, e: NT=not tested, – =original solutions.

Table 2. Inactivation of AIV following direct spraying of HOCl solution for 10 sec

HOCl[a] (ppm)	CT[e]/min	Log10 $TCID_{50}$/m*l*		
		PC[b]	RV[c]	NI[d]
50	0	5.50 ± 0.00	4.17 ± 0.57	1.33 ± 0.57
	3	5.70 ± 0.44	≤ 2.5	≥ 3.2
100	0	5.50 ± 0.00	≤ 2.5	≥ 3.0
200	0	5.62 ± 0.15	≤ 2.5	≥ 3.1

a: HOCl=hypochlorous acid, b: PC=positive control: AIV was mixed with MM, but not HOCl solutions, c: RV=remaining virus after treatment with HOCl solutions, d: NI=neutralization index, e: CT=contact times.

Table 3. Inactivation of AIV with indirect spraying of hypochlorous acid solution for 10 sec and a contact time of 10 min

HOCl[a] (ppm)	Log10 $TCID_{50}$/m*l*		
	PC[b]	RV[c]	NI[d]
50	5.50 ± 0.29	4.50 ± 0.20	1.00 ± 0.2
100	5.96 ± 0.46	4.03 ± 0.60	1.94 ± 0.6
200	5.78 ± 0.26	≤ 2.50	≥ 3.28

DISCUSSION

Chlorine compounds are very popular because they are easy to use and have a wide range of applicability, they can be used quickly, they have a broad spectrum, they are readily available, they have relatively low toxicity to human and animals, and their costs are lower [5, 29]. Their disinfection ability is reduced in the presence of organic materials [5, 17]. Hypochlorites are powerful oxidizing agents with bactericidal, fungicidal and sporicidal activity, and hypochlorous acid is their active moiety [15, 29]. There is less information available concerning the mechanism of action of hypochlorous acid solution, but in general, it affects structural proteins, such as the capsid or surface compounds, lipid envelop (if present) and nucleic acids (DNA or RNA) of viruses [4, 20, 27].

Hypochlorous acid solution is one of the chlorine compounds with good disinfection ability [5, 7, 27]. In the present study, the aqueous phase of the original solution containing a free available chlorine concentration of 50 ppm could reduce the titer of an ordinary AIV (H7N1) from $10^{7.7}$ $TCID_{50}$/m*l* to lower than the detectable limit within 5 sec (Table 1), which is faster than in previous reports [18, 27], and its harvested solution after spraying from a distance of 1 cm had the same ability, but it lost its efficacy after spraying from a distance of 30 cm. Zhao *et al.* also showed similar data; they reported free chlorine loss during spraying depending on the distance [32]. Tamaki *et al.* suggested that the minimum concentration of free chlorine for a virucidal effect of neutral electrolyzed water was approximately 40 ppm [27]. In contrast to 50 ppm solution, 100 or 200 ppm solutions retained their efficacy after travelling the same distance (Table 1). If solutions could not inactivate AIV within 5 sec, they could not inactivate the virus even with 5 min of contact, and this means that their active ingredient (HOCl) was depleted soon after the reaction. In the direct spray form of the solutions, a higher concentration was required; this was probably due to the free chlorine lose during its travel from the nozzle to the sample [32]. The 100 and 200 ppm concentrated solutions inactivated more than 99.9% of AIV directly after spraying, while the 50 ppm concentration required at least 3 min of contact (Table 2). In the indirect spray form (aerosol), as the solution was not sprayed directly onto the inoculated rayon sheet, a lower amount of solution had a chance to come in contact with the AIV present on the sheet, and it required at least 10 min of contact, but it still had higher efficacy, namely, fast and complete inactivation (Table 3), than found in a previous report using a chlorine concentration of 300 ppm [7]. Direct spraying of the solution for a short period of time with a higher concentration will be applicable for disinfection of vehicles or clothes of farm personal or visitors in an airlock entrance just before entering the farms and/or movement from one part of a farm to another, and the present study shows that the efficacy is directly related to the free available chlorine concentration, spray distance from the application area and the exposure time. The ability of a sprayer to make smaller particles may help solution's molecules to be suspended in the air for a longer period of time because of their low settling velocity rate [10], and this may increase its chance to come in contact with pathogens and inactivate them. The presence of many organic materials in the air and/or application surfaces, as well as a long distance between the spray system and application area, might significantly reduce the activity of sprayed hypochlorous acid solutions against pathogens, and these are important factors to be considered during their application on farms.

Installation and application of an appropriate spray system at the entrance (like an airlock entrance) and inside of animal farms at an appropriate distance and use of an ideal disinfectant, such as slightly acidic hypochlorous water, with a proper concentration would potentially reduce the chance of transmission of infections and diseases outbreaks. As farm conditions are totally different from laboratory conditions, further investigation is still required to evaluate sprayed hypochlorous acid solutions for their efficacy.

Lower cost, easy mass applicability, availability and safety are the most useful points that would encourage farmers to use sprayed hypochlorous acid solutions as aerosol disinfectants on their animal farms.

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