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NOTE

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Virucidal activity of a quaternary ammonium compound associated with calcium hydroxide on avian influenza virus, Newcastle disease virus and infectious bursal disease virus

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ABSTRACT. A quaternary ammonium compound (QAC) was evaluated for its virucidal efficacies with food additive grade calcium hydroxide ($FdCa(OH)_2$). When the QAC was diluted 1:500 (QACx500) with redistilled water (dW_2), it inactivated avian influenza virus (AIV) within 30 sec at 25°C, while at 2°C, it required 1 hr for inactivation. When $FdCa(OH)_2$ powder was added to QACx500 at a final concentration of 0.17%, the mixture, namely Mix500, inactivated AIV within 3 min at 2°C. After contamination with 1% fetal bovine serum (FBS), Mix500 inactivated AIV within 2 hr at 2°C, but QACx500 did not. These results indicate synergistic effects of the QAC and $FdCa(OH)_2$ solutions on virucidal activity.

KEY WORDS: food additive grade calcium hydroxide ($FdCa(OH)_2$), poultry viral pathogens, quaternary ammonium compound, synergistic effects, virucide

Since 2003, highly pathogenic avian influenza (HPAI) virus subtype H5N1 has caused massive outbreaks of HPAI in Asian countries [17]. During this time, H5N6, further HPAI viruses of the H5 subtype, and H7N7 have epizootically appeared in East Asia [16]. The Ministry of Agriculture, Forestry, and Fisheries in Japan (MAFF) recommended that farmers enforce biosecurity measures on their farms. However, it has been difficult to keep the HPAI virus out, and 12 HPAI outbreaks involving H5N6 occurred at poultry farms in 2016–2017 [13]. In the winter season from November 2016 to March 2017, more than 200 clinical cases of HPAI virus in wild birds were reported, with the highest prevalence in Japan [5]. To enhance food safety, MAFF announced the "Hazard Analysis Critical Control Point (HACCP) at Livestock Farm Level: Farm HACCP" certification standard in August 2009 [14]. Furthermore, the "Japan Good Agriculture Practice" (JGAP) standards for animal farms were established in April 2017 (Japan GAP Association) [9]. For both systems, the enforcement of biosecurity measures on farms is most important. In addition to avian influenza virus (AIV), other common pathogenic agents, like Newcastle disease virus (NDV), infectious bursal disease virus (IBD), *Salmonella* spp. and *Escherichia coli* should be controlled effectively.

Quaternary ammonium compounds (QACs) are common disinfectants at farms, but their activities are normally reduced by organic material contamination and low temperature [1, 8, 18, 26]. Sodium hypochlorite (NaOCl) is also a popular disinfectant, but its efficacy is limited under organic material contamination conditions [4, 25]. Food additive grade calcium hydroxide ($FdCa(OH)_2$) is relatively novel among materials that can inactivate pathogens [2, 3, 15, 25] and is therefore attractive as a potential biocidal agent. Recently, we found a synergistic effect on bactericidal activity with NaOCl and $FdCa(OH)_2$ [25]. For application at the farm using a sprayer, it is desirable to generate fine particles with diameters less than 10 μm to avoid clogging the sprayer nozzle (Takehara, personal communication).

To enhance biosecurity at farms in winter, proper application of disinfectants is important. In the present study, we evaluate a QAC for its virucidal activity towards AIV, NDV and IBDV, at low temperature and in the presence of organic materials.

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Table 1. The virucidal activities of each tested solution toward AIV under different conditions

Solution	Temp. (°C)	FBS (%)	0 sec	5 sec	30 sec	3 min	30 min	1 hr	2 hr
QACx500[a)]	25	0	8.25 ± 0.00[d)]	6.50 ± 0.20	3.83 ± 0.25	NT	NT	NT	NT
$FdCa(OH)_2$[b)]			8.25 ± 0.15	NT	NT	7.58 ± 0.07	5.50 ± 0.20	5.25 ± 0.31	3.92 ± 0.18
Mix500[c)]			8.00 ± 0.00	5.38 ± 0.19	3.44 ± 0.05	NT	NT	NT	NT
Mix500		1	7.92 ± 0.07	NT	7.33 ± 0.14	3.92 ± 0.30	NT	NT	NT
QACx500		5	8.19 ± 0.20	NT	NT	NT	NT	6.17 ± 0.71	5.17 ± 1.07
$FdCa(OH)_2$			8.19 ± 0.05	NT	NT	NT	7.00 ± 0.12	6.08 ± 0.25	6.50 ± 0.12
Mix500			8.00 ± 0.12	NT	7.67 ± 0.14	6.67 ± 0.25	3.50 ± 0.00	NT	NT
QACx500	2	0	8.25 ± 0.00	NT	NT	NT	6.50 ± 0.54	4.17 ± 0.45	NT
$FdCa(OH)_2$			7.92 ± 0.14	NT	NT	NT	NT	7.67 ± 0.18	7.75 ± 0.00
Mix500			8.33 ± 0.14	7.92 ± 0.30	6.00 ± 0.72	4.75 ± 1.02	NT	NT	NT
QACx500		1	7.50 ± 0.00	NT	NT	NT	6.50 ± 0.00	5.38 ± 0.18	5.25 ± 0.20
Mix500			8.25 ± 0.12	NT	NT	NT	6.58 ± 0/18	6.08 ± 0.34	5.50 ± 0.12
QACx500		5	8.25 ± 0.00	NT	NT	NT	NT	8.00 ± 0.12	8.25 ± 0.12
Mix500			8.00 ± 0.20	NT	NT	NT	NT	7.08 ± 0.34	7.00 ± 0.35

a) A quaternary ammonium compound (QAC) diluted 1:500 (QACx500). b) Food additive grade calcium hydroxide powder (170 mg) was prepared in 100 m*l* of redistilled water ($FdCa(OH)_2$). c) $FdCa(OH)_2$ powder (170 mg) was prepared in 100 m*l* of QACx500 (Mix500). d) Virus titer ($TCID_{50}$/m*l*). NT: Not tested.

Inactivation of AIV was also tested at room temperature, due to the low efficacy of the QAC toward AIV under low temperature conditions in the present experiments. The synergistic effects of QAC and $FdCa(OH)_2$ were also examined.

The QAC (Rontect®) was kindly supplied by Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) and was diluted 1:500 (QACx500) with redistilled water (dW_2), to obtain a final concentration of 200 ppm didecyl-dimethylammonium chloride (DDAC), as recommended by the manufacturer. $FdCa(OH)_2$ powder at pH 13 was made of natural calcium carbonates derived from limestone through a calcination process, with the average diameter of the powder particles being 10 μm [3, 25]. It was kindly provided by Fine Co., Ltd. (Tokyo, Japan). A quantity of 170 mg of $FdCa(OH)_2$ powder was added to 100 m*l* of dW_2 or 100 m*l* of QACx500 and centrifuged at 1,750 × g for 10 min at 4°C. The resulting supernatants were used as saturated 0.17% $FdCa(OH)_2$ ($FdCa(OH)_2$ solution) or QAC and $FdCa(OH)_2$ mixture (Mix500), respectively. For room temperature experiments, all solutions were kept at room temperature for at least 30 min. Room temperature was maintained at 25 ± 2°C using an air-conditioner. For low temperature experiments, all solutions and viruses were kept on ice for at least 30 min until the temperature of the solutions became 2 ± 0.5°C. These solution temperatures were confirmed with bar thermometers.

Low pathogenic avian influenza (LPAI) virus A/duck/Aomori/395/04 (H7N1) [7], NDV strain Sato [20], and IBDV vaccine strain D78 (Intervet Co., Ltd., Tokyo, Japan) [21] were used. AIV and NDV were propagated in 10-day-old embryonated chicken eggs. AIV was titrated in Madin-Darby canine kidney (MDCK) cells in the form of 50% tissue culture infectious dose ($TCID_{50}$) [6], which was found to be 108.2 $TCID_{50}$/m*l*. NDV and IBDV were titrated in chicken embryo fibroblasts (CEF) prepared from 10-day-old chicken embryos in the form of $TCID_{50}$/m*l* for NDV [23] (found to be $10^{7.2}$ $TCID_{50}$/m*l*), and plaque-forming units (PFU)/m*l* for IBDV [21] (found to be $10^{5.3}$ PFU/m*l*).

Four hundred microliters of QACx500, $FdCa(OH)_2$ solution, or Mix500 were mixed with 100 μl of the viruses, AIV, NDV, or IBDV, in a microtube and incubated for the indicated time (0 sec, 5 sec, 30 sec, 3 min, 30 min, 1 hr or 2 hr) on ice. The reaction was then stopped by adding 500 μl of a blocking solution (30% FBS in 0.7 M HEPES, pH 7.2) [14]. To confirm the effect of the blocking solution, the tested solutions were mixed with blocking solution before adding the virus (referred to as treatment of "0" sec). To evaluate the inactivating activity of the tested solutions in the presence of organic materials, 5 or 25 μl of FBS was added to 395 or 375 μl of the solutions, respectively, and then 100 μl of virus was added. This resulted in FBS concentrations of 1 or 5%, respectively, in microtubes containing the virus. For AIV inactivation evaluation, reactions were also performed at room temperature. However, for NDV and IBDV, reactions were performed only at cold temperature. Each solution was tested in triplicate and the titers are shown as mean ± standard error (SE).

Inactivation efficacy against the viruses was determined by calculating the reduction factor (RF), using the following equation: RF=tpc−ta, where tpc is the titer of the untreated sample in $\log_{10}$ units and ta is the titer of the recovered virus of the treated samples. Inactivation was considered to be satisfied when RF was ≥3, indicating a reduction of virus titer greater than 1,000 times [11, 19, 24].

When the blocking solution was added to the solution before adding the virus (0 sec), no or minor reductions of virus titer were observed (Tables 1 and 2). As shown in Table 1, at 25°C, QACx500 reduced AIV titer more than 1,000 times, with AIV inactivation occurring within 30 sec (RF=4.08) in the absence of FBS. However, in the presence of 5% FBS, QACx500 did not inactivate AIV within 2 hr. The $FdCa(OH)_2$ solution required 2 hr to inactivate AIV at 25°C, with no FBS. The Mix500 solution inactivated AIV within 5 sec, 3 and 30 min in the presence of FBS at 0, 1 and 5%, respectively. At 2°C, the virucidal activity of QACx500 was reduced. At this temperature, QACx500 required 1 hr to inactivate AIV in the absence of FBS and it did not inactivate AIV within 2 hr in the presence of 1 or 5% FBS. The $FdCa(OH)_2$ solution did not inactivate AIV at 2°C, either in the

Table 2. The virucidal activities of each tested solution toward NDV and IBDV at 2°C

Virus	Solution	FBS (%)	0 sec	5 sec	30 sec	3 min	30 min	1 hr
NDV	QACx500[a)]	0	6.75 ± 0.12[d)]	4.38 ± 0.27	3.38 ± 0.27	NT	NT	NT
	$FdCa(OH)_2$[b)]		7.00 ± 0.20	NT	NT	5.58 ± 0.18	4.00 ± 0.12	NT
	Mix500[c)]		6.42 ± 0.18	2.92 ± 0.25	2.83 ± 0.27	NT	NT	NT
	Mix500	5	7.17 ± 0.36	NT	5.33 ± 0.83	3.92 ± 0.18	NT	NT
IBDV	QACx500	0	4.54 ± 0.18[e)]	NT	NT	3.23 ± 0.41	3.27 ± 0.44	3.20 ± 0.08
	$FdCa(OH)_2$		5.19 ± 0.30	1.7 ± 0.14	1.4 ± 0.00	1.4 ± 0.00	NT	NT
	Mix500		4.48 ± 0.24	1.40 ± 0.00	1.56 ± 0.13	NT	NT	NT
	Mix500	5	4.33 ± 0.28	1.40 ± 0.00	1.40 ± 0.00	NT	NT	NT

a) A quaternary ammonium compound (QAC) diluted 1:500 (QACx500). b) Food additive grade calcium hydroxide powder (170 mg) was prepared in 100 m*l* of redistilled water ($FdCa(OH)_2$). c) $FdCa(OH)_2$ powder (170 mg) was prepared in 100 m*l* of QACx500 (Mix500). d) Virus titer ($TCID_{50}$/m*l*). e) Virus titer (PFU/m*l*). NT: Not tested.

absence or presence of FBS. Mix500 inactivated AIV within 3 min or 2 hr in the absence or presence of 1% FBS, respectively, at 2°C. However, it did scarcely inactivate AIV in the presence of 5% FBS (Table 1).

The virucidal activities of all tested solutions towards NDV and IBDV at 2°C are shown in Table 2. In the absence of FBS, QACx500 inactivated NDV within 30 sec, $FdCa(OH)_2$ within 30 min, and Mix500 within 5 sec. In the presence of 5% FBS, Mix500 inactivated NDV within 30 sec. At 2°C, IBDV was inactivated within 5 sec by every solution, except for QACx500, either in the absence or presence of 5% FBS.

QACs are used widely at poultry farms in car gate sprays, equipment washing solutions, foot baths, or premises washing solutions; however, their biocidic efficacies in cold weather are decreased [8, 18, 26]. Here, we established a strategy for QAC utility under cold conditions, in the presence of organic materials. Our results demonstrate that QAC activities can be synergistically enhanced with a $FdCa(OH)_2$ solution (Tables 1 and 2). At 2°C, in the absence of FBS, QACx500 required 1 hr to inactivate AIV and $FdCa(OH)_2$ could not inactivate the virus within 2 hr, but Mix500 was effective within 3 min. However, in the presence of a high concentration of organic material (5% FBS), even Mix500 could scarcely inactivate AIV, as shown in Table 1. The importance of a cleaning/washing step for proper disinfection is well known, and this was confirmed here, even with Mix500. The synergistic effect was also observed toward NDV during the required period for inactivation of 30 sec for QACx500 and 5 sec for Mix500 (Table 2). However, the effect on NDV was not as significant as the effect on AIV. QAC did not inactivate IBDV, a non-enveloped virus, but the $FdCa(OH)_2$ solution did inactivate this virus within 5 sec (Table 2). It has been shown that IBDV is easily inactivated under high alkali conditions [21, 22].

QACs are membrane-active agents that interact with the cytoplasmic membrane and intracellular targets [1]. The cytoplasmic membrane is a lipid bilayer formed by fatty molecules called phospholipids. At lower temperatures, the fatty acid tails of these phospholipids move less and become more rigid, which makes the cytoplasmic membrane resistant to QACs. The calcium ion (Ca^{2+}) has been shown to enhance the transfection potency of plasmid DNA-cationic liposome complexes [10]. It is likely, though not yet proven, that the mechanism of this synergistic effect seems to be through high pH and calcium ion, which made the viral membrane damaged, allowing QAC to pass the membrane and consequently interact with intracellular targets. We propose a similar synergistic mechanism between NaOCl and $FdCa(OH)_2$, in that high pH and calcium damaged the bacterial cell membrane [12], thereby enabling OCl^- ions to pass through and denature proteins in the cytosol of bacterial cells [25].

In conclusion, these results demonstrate that the QAC and $FdCa(OH)_2$ solutions have synergistic effects, resulting in a more effective virucidal agent, with a wide spectrum of pathogen inactivation.

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