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FULL PAPER

Avian Pathology

Synergistic effects of quaternary ammonium compounds and food additive grade calcium hydroxide on microbicidal activities at low temperatures

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ABSTRACT. The microbicidal activities of mixtures of quaternary ammonium compounds (QACs) and food additive grade calcium hydroxide ($FdCa(OH)_2$) were evaluated in a suspension test at −20°C using an anti-freeze agent (AFA) containing methanol, or at 1°C, with varying contact time, toward avian influenza virus (AIV), Newcastle disease virus (NDV), fowl adenovirus (FAdV), avian reovirus (ARV), *Salmonella* Infantis (SI) and *Escherichia coli* (EC). At −20°C, the mixtures could inactivate AIV and NDV within 30 min, FAdV and ARV within 5 sec, and SI and EC within 3 min, respectively. AFA did not inactivate viruses and bacteria within 30 min and 10 min, respectively. At 1°C, the mixtures inactivated FAdV and ARV within 30 sec, AIV within 10 min, and NDV within 30 min. A mixture of slaked lime (SL) and QAC could inactivate FAdV and ARV within 30 sec, but could not inactivate AIV and NDV even after 60 min at 1°C. SL could not substitute $FdCa(OH)_2$ in order to exert the synergistic effects with QAC. Thus, QACs microbicidal activities were maintained or enhanced by adding $FdCa(OH)_2$. It is hence recommended to use QACs with $FdCa(OH)_2$, especially in the winter season.

KEY WORDS: calcium hydroxide, low temperature, quaternary ammonium compound, slaked lime, synergistic effect

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Enhancement of farm biosecurity through the use of appropriate disinfectants is one of the most important means of reducing the amount of pathogens [20, 36]. Prevention and control of diseases largely depend on biosecurity, and disinfectants are essential tools for biosecurity program [1, 11, 33]. Various environmental factors such as temperature, organic load and short contact time can reduce disinfection abilities [3, 9, 17, 28, 32]. The efficacies of some disinfectants will be lost at low temperatures [2, 5, 14].

Poultry infectious diseases, especially avian influenza (AI), Newcastle disease (ND), infectious bursal disease (IBD), the diseases due to fowl adeno virus (FAdV) and avian reovirus (ARV), colibacillosis and salmonellosis are highly contagious and detrimental for the poultry industry. Since 2003, especially during the winter season, highly pathogenic avian influenza (HPAI) has been widespread and persistent in Asia and Africa, and discontinuously in Europe [24, 27]. NDV is one of the notorious poultry pathogens severely endangering out the poultry industry and resulting in a highly contagious septicemic, fatal, and destructive disease that affects wide varieties of domestic and wild birds worldwide [6, 7]. FAdV and ARV are ubiquitous in poultry facilities because of their resistance to wide ranges of disinfectants [8, 13, 23, 26].

Quaternary ammonium compounds (QACs) are common disinfectants widely used at livestock farms and food processing industries, owing to their relatively low toxicity and broad antimicrobial spectrum, alongside with chemical stability. However, their inactivation efficacies are diminished by organic material contamination or at low temperatures [9, 17, 28] and QACs are not able to inactivate non-enveloped viruses [8, 14].

The synergistic virucidal activity of $FdCa(OH)_2$ and QAC was shown in the suspension test at 2°C, and the spectrum of virucidal activity of QAC with addition of $FdCa(OH)_2$ was also shown to be broadened against a non-enveloped virus [14].

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The synergistic virucidal and bactericidal activities at 2°C were also shown in the suspension and carrier tests [1, 2]. It was also demonstrated that FAdV and ARV were inactivated within 5 sec by $FdCa(OH)_2$ regardless of the presence or absence of organic matter and temperature conditions (25°C and 2°C) [4]. Guan *et al.* showed that preparations of disinfectant supplemented with methanol (MeOH) as anti-freeze agent could be effectively applied at temperatures as low as −20°C against AIV for disinfection [10].

Quite recently, in the evaluation of disinfectants, it has become clear that some disinfectants could inactivate pathogens in suspension tests, but not in carrier tests [21, 22]. For the evaluation of disinfectants, the suspension test is the first screening and the carrier test is the second screening for the field usages [22, 25].

Slaked lime (SL), an inorganic compound containing >65% $Ca(OH)_2$ and a strong alkaline substance was also used to inactivate pathogens [35]. In Japan, the Ministry of Agriculture, Forestry and Fisheries (MAFF) recommends to livestock farmers to spread SL powder for standby sterilization [29]. Livestock farmers tend to use SL powder as a disinfectant for foot baths to inactivate viruses and bacteria on their boots during freezing season, or try to use it in combination with QACs in the hope of replacing $FdCa(OH)_2$ (Takehara, personal communication). However, it takes 3 to 6 hr for the SL powder to inactivate pathogens [12], and its synergistic effects with QACs has not been demonstrated at saturated conditions.

It is very important to keep disinfectants efficacies even in cold conditions. In the present study, the synergistic efficacies of QACs with alkaline agents such as $FdCa(OH)_2$ and SL were examined at low temperatures. It is also important to perform the experiments at the indicated temperature, for example, using aluminum racks to maintain the exact temperature as shown in Fig. 1. Otherwise, the temperature will become high and will not get the correct data.

MATERIALS AND METHODS

Experimental design

We evaluated the synergistic microbicidal efficacies of the mixtures of $FdCa(OH)_2$ and QACs or SL and QAC toward several viruses and bacteria, especially at low temperatures during the winter season for proper selection and dose maintaining of the disinfectants. Suspension tests were conducted with these disinfectants and evaluated for several contact times for both viruses and bacteria. For the experiments at −20°C, anti-freezing agent was mixed with equal volume of disinfectant solutions to avoid freezing. After disinfectants treatments, the remained viruses or bacteria were titrated.

Anti-freeze agent

A commercially available window washer, Kaihyo Super washer solution® made by KOGA Chemical Mfg Co., Ltd. (Saitama, Japan), contains 51–53wt % MeOH along with surfactants, chelator and antiseptic agents was purchased at a local market and used as the anti-freeze agent (AFA) that lowers the freezing point of a water-based liquid. In this study, for the experiments at −20°C, QACs were diluted 1:250 with 0.34% $FdCa(OH)_2$ and mixed with AFA in equal amounts.

Chemical disinfectants and neutralizers

QACs, Rontect® containing didecyl dimethyl ammonium chloride and Pacoma® containing trimethyl ammonium chloride were purchased from Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) and Meiji Seika Pharma Co. (Tokyo, Japan), respectively. $FdCa(OH)_2$ powder with average particle diameter 10 µm was kindly supplied by Fine Co., Ltd. (Tokyo, Japan). Rontect® and Pacoma® were diluted 500 times with 0.17% $FdCa(OH)_2$ (thereafter, Rox500 and Pax500, respectively). For −20°C experiments, these QACs were diluted 1:250 with 0.34% $FdCa(OH)_2$ and mixed equally with AFA (finally 1:500 dilution for QACs and 0.17% $FdCa(OH)_2$: thereafter, Rox500-20 or Pax500-20, respectively). As negative control, AFA mixed with phosphate buffer saline (PBS) in equal amount was prepared and designated as AFA-PBS-20. SL of 70% calcium hydroxide, 70.0 Slaked lime® (made in China, imported by Kumamoto Bussan Co., Kumamoto, Japan), with particle diameter less than 150 µm was purchased.

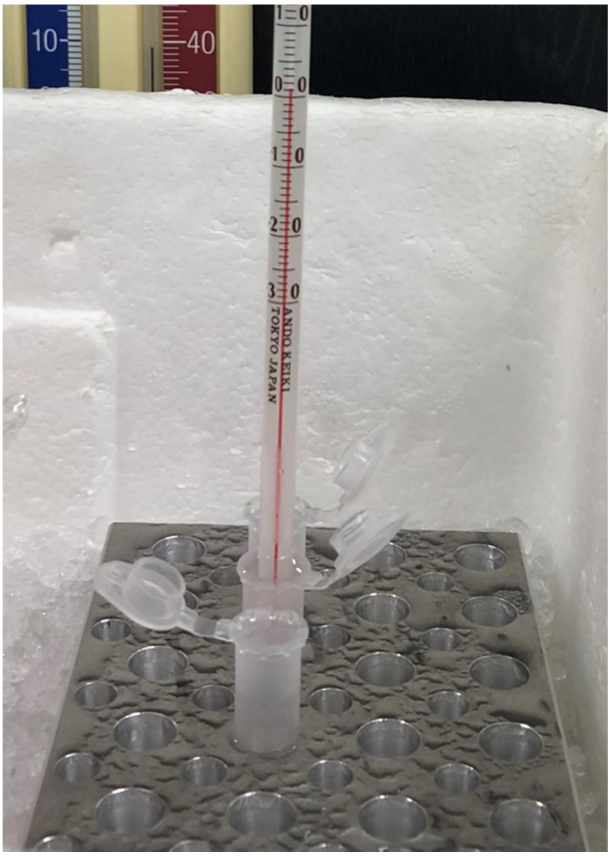

Fig. 1. An aluminum rack kept on ice. To ensure temperatures at 1°C or at −20°C, all reactions were performed in aluminum racks. For the 1°C experiments, the racks were placed on ice; for the −20°C experiments, the racks were kept in a −20°C freezer and removed from the freezer to a safety cabinet on ice. After solutions were added, the racks were immediately returned to the freezer.

Virucidal efficacies of QAC with 0.24% SL (solid SL 0.24 g and 200 µl Rontect® added in 100 ml dW_2 defined as Ron+SL) and single 0.24% SL (solid SL 0.24 g added in 100 ml dW_2 designated as saturated SL) were also evaluated. For the experiments at 1°C, all solutions and viruses were kept on ice for around 1 hr until the solution's temperature became 1°C before starting the experiments as shown in Fig. 1. For experiments at −20°C, all solutions containing 50% AFA were kept at −20°C freezer for at least 1 hr until the temperature became −20°C before starting the experiments. These solutions temperatures were confirmed with a bar thermometer and during the experiment, all microtubes were placed in aluminum racks and temperature was strictly controlled (Fig. 1).

Chemical neutralizer, namely a blocking solution for virus (thereafter BSV) containing 30% fetal bovine serum (FBS) in 0.7 M 4-(2-hydroxyl)-1-piperazineethanesulfonic acid (pH 7.2), was prepared for neutralizing the virus inactivation efficacy of solutions right after the given disinfectant contact time [1]. Another blocking solution for bacteria (thereafter BSB) containing 30% FBS in 0.7 M Tris–HCl (pH 7.2) to stop the bacterial inactivation reaction was prepared and used right after the selected contact time of disinfectant application [2].

Viruses, bacteria and cells

A low pathogenic AIV, A/duck/Aomori/395/04 (H7N1) [16] and virulent NDV strain Sato [31] as enveloped viruses, and FAdV strain Ote and ARV strain Uchida [4] as non-enveloped viruses were prepared and titrated in the form of 50% tissue culture infective dose ($TCID_{50}$)/ml [15] in primary chicken kidney (CK) cells. Salmonella Infantis (SI), *Escherichia coli* (EC) were grown in Luria Bertani (LB) medium and titrated on DHL agar as colony forming units (CFU)/ml as described [2].

Evaluation of the virucidal and bactericidal activities using the suspension test

In the present study, two reaction temperatures (−20°C and 1°C) and several contact time points were used for investigating the inactivation process of the above-mentioned viruses and bacteria in the aqueous phase using the suspension test. For −20°C experiment with viruses and bacteria, all microtubes containing 400 µl of disinfectants were kept at −20°C freezer for 1 hr. And for the 1°C investigation, all microtubes containing 400 µl of disinfectants were kept on ice for around 1 hr.

Four hundred microliters of Rox500-20, Pax500-20 or AFA-PBS-20 were mixed separately with 100 µl of each virus, then incubated for indicated times at −20°C. In parallel, 400 µl of Rox500-20 or Pax500-20 were mixed with 500 µl of BSV in a microtube and then was added 100 µl of each viral solution to the microtube, to evaluate the blocking solution's effect (shown as 0 sec treatment and contact time).

At 1°C, microtubes containing 400 µl Rox500, Pax500, Ron+SL or saturated SL were mixed with 100 µl of each virus, and then incubated for the indicated times, in parallel with 0 sec.

After the process of incubation, the virus inactivation efficacy of each solution was stopped by adding 500 of BSV. The remaining viable virus titer in each sample, including the positive control that 100 µl of each virus was mixed with 200 µl of PBS, 200 µl of AFA and 500 µl of BSV and then kept at 1°C for 1 hr, was titrated (log $TCID_{50}$/ml) by inoculating it on CK cells in 96-well tissue culture plates (four wells per dilution, 200 µl final volume in each well) after making serial 10-fold dilutions.

For bactericidal evaluation, 400 µl of Rox500-20 or AFA-PBS-20 was mixed with 100 µl of each bacterial solution, and then incubated at −20°C for 3 or 5 min for Rox500-20 and for 5 or 10 min for AFA-PBS-20. After incubation, the bactericidal efficacy of the tested solution was blocked by adding 500 µl of BSB. At last, bacterial viable counting in each sample was calculated (log_{10} CFU/ml) by plating 25 µl portions on DHL agar plates after making serial ten-fold dilutions in PBS, followed by 24 hr incubation at 37°C.

In the case of positive control, 100 µl of bacteria were separately mixed with 200 µl of PBS, 200 µl of AFA and 500 µl of BSB kept at 1°C for 1 hr, and then ten-fold serial dilutions were made.

The tested solution was evaluated in triplicate, and the titers were shown as mean ± SE. Inactivation was considered to be effective if ≥3 log_{10} reduced organism titers were obtained, indicating a more than 1,000 times viral or bacterial titer reduction [18, 30, 33].

RESULTS

Evaluation of the virucidal activities of the solutions at −20°C

Table 1 shows the virucidal activities of Rox500-20, Pax500-20 and AFA-PBS-20 toward AIV, NDV, FAdV and ARV at −20°C. Rox500-20 was able to reduce the titers of enveloped viruses, namely AIV and NDV within 30 min and of non-enveloped viruses, namely FAdV and ARV, within 30 sec. Pax500-20 was almost as effective as Rox500-20, but slightly inferior. No viral inactivation was detected at 0 sec, where neutralizing solution was added before virus addition, compared to the control viruses. AFA-PBS-20 had no virucidal effectiveness toward AIV, NDV, FAdV and ARV within 30 min at −20°C.

Evaluation of the virucidal activities of the solutions at 1°C

Table 2 shows the virucidal activities of Rox500, Pax500, Ron+SL, and saturated SL towards AIV, NDV, FAdV and ARV at 1°C. Rox500 and Pax500 were able to inactivate FAdV, ARV within 30 sec, AIV within 10 min and NDV within 30 min, respectively at effective level (≥3 log_{10} $TCID_{50}$/ml). Ron+SL and saturated SL could not inactivate AIV and NDV even after 60 min. However, Ron+SL could inactivate FAdV and ARV within 30 sec.

Evaluation the bactericidal activities of Rox500-20 and AFA-PBS-20 solution at −20°C

Table 3 shows the bactericidal activities of Rox500-20 and AFA-PBS-20 towards SI and EC at −20°C. Rox500-20 was able to inactivate EC to undetectable level (<2.6 $\log_{10}$ CFU/ml) within 3 min and SI in effective level (≥3 log10 CFU/ml) within 3 min and to undetectable level within 5 min. AFA-PBS-20 had no significant bactericidal effectiveness toward SI and EC even after 10 min.

Table 1. Virucidal efficacies of Rox500-20, Pax500-20 and AFA-PBS-20 at −20°C

Solution type	Virus	Control	Viral titer ($\log_{10}$TCID$_{50}$/ml) at different contact times				
			0 sec	5 sec	1 min	10 min	30 min
Rox500-20[a]	AIV	NT	8.66 ± 0.07	7.50 ± 0.00	7.33 ± 0.35	7.00 ± 0.00	3.50 ± 0.00[d]
Pax500-20[b]		NT	8.25 ± 0.11	7.00 ± 0.20	6.91 ± 0.07	5.50 ± 0.18	4.50 ± 0.00[d]
Rox500-20	NDV	NT	9.33 ± 0.07	8.50 ± 0.00	8.00 ± 0.17	6.67 ± 0.47	6.16 ± 0.59[d]
Pax500-20		NT	9.08 ± 0.18	7.41 ± 0.20	7.58 ± 0.24	6.67 ± 0.18	5.50 ± 0.12[d]
Rox500-20	FAdV	NT	8.50 ± 0.28	4.25 ± 0.38[d]	3.50 ± 0.00[d]	NT	NT
Pax500-20		NT	8.58 ± 0.30	3.50 ± 0.17[d]	3.17 ± 0.33[d]	NT	NT
Rox500-20	ARV	NT	8.91 ± 0.08	3.58 ± 0.65[d]	3.75 ± 0.25[d]	NT	NT
Pax500-20		NT	8.91 ± 0.08	4.58 ± 0.50[d]	3.42 ± 0.30[d]	NT	NT
AFA-PBS-20[c]	AIV	8.50 ± 0.28	NT	NT	NT	NT	7.58 ± 0.13
	NDV	9.25 ± 0.32	NT	NT	NT	NT	8.50 ± 0.20
	FAdV	8.25 ± 0.00	NT	NT	NT	NT	8.00 ± 0.00
	ARV	8.67 ± 0.07	NT	NT	NT	NT	8.25 ± 0.11

[a] Quaternary ammonium compounds (QAC) -Rontect® from Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) was diluted 1:250 with 0.34% FdCa(OH)$_2$ and mixed equally with the anti-freeze agent (AFA). [b] QAC-Pacoma®, Meiji Seika Pharma Co. (Tokyo, Japan) was diluted 1:250 with 0.34% FdCa(OH)$_2$ and mixed equally with the AFA. [c] The anti-freeze agent (AFA) mixed with phosphate buffered saline (PBS) in equal amount as AFA-PBS-20. [d] Effective viral inactivation if ≥3 $\log_{10}$ tissue culture infective dose $_{50}$/ml (TCID$_{50}$/ml). Data represent means ± standard error of 3 different experiments. 'NT' denoted as not tested.

Table 2. Virucidal efficacies of Rox500, Pax500, Ron + SL and saturated SL at 1°C

Solution type	Virus	Viral titer ($\log_{10}$TCID$_{50}$/ml) at different contact times							
		0 sec	5 sec	30 sec	1 min	3 min	10 min	30 min	60 min
Rox500[a]	AIV	8.91 ± 0.24	NT	NT	6.08 ± 0.07	NT	4.41 ± 0.37[e]	4.16 ± 0.07[e]	3.75 ± 0.11[e]
Pax500[b]		8.58 ± 0.16	NT	NT	6.41 ± 0.13	NT	4.67 ± 0.07[e]	4.33 ± 0.13[e]	3.58 ± 0.07[e]
Rox500	NDV	9.25 ± 0.00	NT	NT	7.08 ± 0.07	NT	7.00 ± 0.23	4.67 ± 0.36[e]	4.58 ± 0.49[e]
Pax500		9.62 ± 0.15	NT	NT	8.00 ± 0.50	NT	7.50 ± 0.35	4.83 ± 0.36[e]	4.25 ± 0.50[e]
Ron+SL [c]	AIV	7.83 ± 0.068	NT	NT	6.17 ± 0.29	6.08 ± 0.136	NT	NT	5.67 ± 0.07
	NDV	9.08 ± 0.068	NT	7.50 ± 0.00	7.50 ± 0.00	7.50 ± 0.00	NT	NT	6.33 ± 0.07
	FAdV	7.07 ± 0.068	5.25 ± 0.00	3.75 ± 0.71[e]	NT	NT	NT	NT	NT
	ARV	8.41 ± 0.068	5.16 ± 0.07[e]	3.83 ± 0.27[e]	NT	NT	NT	NT	NT
Saturated SL[d]	AIV	7.83 ± 0.068	NT	NT	7.50 ± 0.00	NT	7.50 ± 0.00	7.50 ± 0.00	7.25 ± 0.07
	NDV	9.08 ± 0.068	NT	NT	7.50 ± 0.00	NT	7.50 ± 0.00	7.50 ± 0.00	6.91 ± 0.13

[a] Quaternary ammonium compounds (QAC) -Rontect® from Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) was diluted 1:500 with 0.17% FdCa(OH)$_2$. [b] QAC (Pacoma®), Meiji Seika Pharma Co. (Tokyo, Japan) was diluted 1:500 with 0.17% FdCa(OH)$_2$. [c] Solid slaked lime 0.24 g and 200 µl QAC-Rontect® added in 100 ml dW$_2$. [d] Solid slaked lime 0.24 g added in 100 ml dW$_2$. [e] Effective viral inactivation if ≥3 $\log_{10}$ tissue culture infective dose $_{50}$/ml (TCID$_{50}$/ml). Data represent means ± standard error of 3 different experiments. 'NT' denoted as not tested.

Table 3. Bactericidal efficacies of Rox500-20 and AFA-PBS-20 at −20°C

Solution Type	Bacteria	Bacterial titer $\log_{10}$CFU/ml at different contact times			
		Control	3 min	5 min	10 min
Rox500-20[a]	*Escherichia coli*	8.60 ± 0.00	<2.6 ± 0.00[c]	<2.6 ± 0.00[c]	NT
	Salmonella Infantis	9.00 ± 0.00	5.57 ± 0.09[d]	<2.6 ± 0.00[c]	NT
AFA-PBS-20[b]	*E. coli*	8.09 ± 0.40	NT	8.09 ± 0.36	7.91 ± 0.08
	S. Infantis	8.24 ± 0.39	NT	8.24 ± 0.05	8.18 ± 0.28

[a] QAC-Rontect® was diluted 1:250 with 0.34% FdCa(OH)$_2$ and mixed equally with the anti-freeze agent. [b] The anti-freeze agent (AFA) mixed with PBS in equal amount as AFA-PBS-20. [c] Undetectable level, when <2.6 $\log_{10}$ colony forming unit (CFU/ml). [d] Effective bacterial inactivation (≥3 $\log_{10}$ (CFU/ml). Data represent means ± SE of 3 different experiments.

DISCUSSION

Disinfection is essential for breaking down the infection chain of pathogens by reducing the risk of cross-contamination. Disinfectants are used for disease prevention and control [20]. The efficacies of most chemical disinfectants are affected by the presence of organic materials, low temperatures, and time of contact with pathogens [9, 17, 19].

QACs are common disinfectants widely used in livestock farms and food processing industries, and their weakness, the decrease in inactivation activity at low temperature, has been overcome by the synergistic virucidal and bactericidal activities generated through the addition of $FdCa(OH)_2$ at 2°C [1, 2, 14].

Thus, we evaluated changes in the efficacies of combined disinfectants used in this study under different contact times, at very low temperature (−20°C), and at low temperature (1°C).

In the present study, the QACs and $FdCa(OH)_2$ mixtures with the MeOH based AFA namely Rox500-20 and Pax500-20, showed synergistic activity even at −20°C, as described previously for 2°C [1, 2, 14]. Pacoma® is the 2nd and Rontect® is the 3rd generations of QACs [9], however as shown in Table 1, their virucidal activities with $FdCa(OH)_2$ were not much different (Pacoma® was slightly inferior).

When the slaked lime powder was added to QAC in order to reach a saturated (0.17%) solution; so while the original slaked lime has 70% $Ca(OH)_2$ (and not 0.17g), 0.24 g slaked lime powder was added to 100 ml of 1:500 diluted Rontect® to make 0.17% saturated solution−instead of $FdCa(OH)_2$ at 1°C-the synergistic effects were not demonstrated with QAC (Table 2). The mixture with Rontect® and Pacoma® with $FdCa(OH)_2$, namely Rox500 and Pax500 inactivated AIV in 10 min and NDV in 30 min, respectively (Table 2). The differences between $FdCa(OH)_2$ and the slaked lime were purity of $Ca(OH)_2$ and particle size; the $Ca(OH)_2$ content of $FdCa(OH)_2$ was 98% and that of the slaked lime was about 70%, and the particle size of the powder was 10 µm for $FdCa(OH)_2$ and less than 150 µm for the slaked lime as shown in MATERIALS AND METHODS. Particle size seems to be important for inactivation activity as previously described [34].

The mixture with Rontect®, $FdCa(OH)_2$, namely Rox500-20 could inactivate EC and SI at −20°C as shown in Table 3. AFA itself as AFA-PBS-20, with the final MeOH concentration about 26%, did not decrease titers in 30 min and 10 min toward the above viruses and bacteria, respectively as shown in Tables 1 and 3. Guan *et al*. showed that MeOH as the cryoprotectant had no adverse effects and that treatment with 20% MeOH alone for up to 30 min did not kill AIV [10].

It is recommended for enhancing farm biosecurity to use $FdCa(OH)_2$ for synergistic and broaden spectrum of QACs in all season, as the mixtures can inactivate not only bacteria but also enveloped and non-enveloped viruses. Besides, it is not recommended to use QACs together slaked lime.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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