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Original Article

Effects of Several Virucidal Agents on Inactivation of Influenza, Newcastle Disease, and Avian Infectious Bronchitis Viruses in the Allantoic Fluid of Chicken Eggs

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SUMMARY: General theories on the inactivation of viruses in the presence of a concentrated protein, such as the allantoic fluid of chicken eggs, are not useful. That is, although sodium hypochlorite and sodium hydroxide are generally known as strong virucidal agents, they do not sufficiently inactivate viruses in allantoic fluid. We found that benzalkonium chloride (BC) is an effective virucidal agent against influenza, Newcastle disease, and avian infectious bronchitis viruses even in the presence of a concentrated protein. BC is easily biodegradable by activated sludge and is not very harmful to humans. We strongly recommend BC as a useful virucidal agent, especially in the manufacture of vaccines for these viruses.

INTRODUCTION

The influenza virus (FluV), Newcastle disease virus (NDV), and avian infectious bronchitis virus (IBV) are categorized as the *Orthomyxoviridae*, the *Paramyxoviridae*, and the *Coronaviridae*, respectively. They are single-stranded ribonucleic acid (RNA) viruses with envelopes. In the manufacture of FluV, NDV, and IBV vaccines, these viruses are propagated in the allantoic cavities of chicken eggs. Therefore, in the manufacturing process, a large amount of highly infectious allantoic fluid is produced and many instruments become highly contaminated by these viruses. However, using virucidal agents, it is not easy to inactivate the viruses in such a large amount of protein waste material such as allantoic fluid (1).

A few of the commonly used virucidal agents are sodium hypochlorite (SHC), sodium hydroxide (SH), and ethanol. SHC and ethanol are known to be virucidal agents that are stronger than benzalkonium chloride (BC), a quaternary ammonium compound (2-4). However, SHC is more toxic to humans than ethanol or BC (4-6). In many of the studies on the effects of virucidal agents conducted thus far, a concentrated protein such as allantoic fluid was not used (7). Therefore, it is questionable whether such virucidal agents are effective for the inactivation of highly infectious FluV, NDV, and IBV in the presence of a concentrated protein such as allantoic fluid. Therefore, we quantitatively studied the effect of several virucidal agents against these three viruses (FluV, NDV, and IBV), which were placed in the allantoic fluid of chicken eggs.

MATERIALS AND METHODS

Viruses: Viruses used for this study were strains that were used to manufacture the FluV, NDV, and IBV vaccines. For the FluV vaccine strains, the A/Beijing/262/95 (H1N1) strain (8) and the B/Guangdong/05/94 strain (9) were used. Both strains were provided by the National Institute of Infectious Diseases of Japan. Moreover, the NIBRG-14 strain of FluV, a candidate strain of a mock-up pandemic FluV vaccine in Japan, was used. This strain is a reassortant prepared by reverse genetics from the A/Viet Nam/1194/2004 (H5N1) virus (from which the polybasic hemagglutinin cleavage site has been excised) and the A/PR/8/34 (H1N1) virus provided by the National Institute of Biological Standards and Control (10). The NDV vaccine strain used was the Ishii strain, provided by the National Veterinary Assay Laboratory of Japan (11). The IBV vaccine strains used were the TM-86 strain, which was isolated from infected chickens at the Chemo-Sero-Therapeutic Research Institute, and the Nerima strain, which was provided by the National Institute of Animal Health of Japan (11). These viruses are able to propagate in the allantoic cavities of chicken eggs.

Virucidal agents: BC, SHC, SH, and ethanol were used for this study as the virucidal agents. The examined concentrations of each virucidal agent are shown in Tables 1-4.

Inactivation treatment of allantoic fluids containing FluV, NDV, and IBV: Two different inactivation methods for allantoic fluids containing FluV, NDV, and IBV were investigated: the adding method and the wiping method.

The adding method: In actual vaccine manufacturing, infectious waste and instruments are steeped in a large amount of antiseptic solution containing virucidal agents. However, in this research, we added the virucidal agents to undiluted allantoic fluid containing one of the three viruses, anticipating the worst case, in order to study the effects of the virucidal agents. For this method, FluVs (A/Beijing/262/95, B/Guangdong/05/94 and NIBRG-14 strains), NDV (Ishii strain), and IBVs (TM-86 and Nerima strains) were used. To make samples of several concentrations as shown in Tables 1-3, we added 10% of BC, 12% of SHC, and 10% of SH into the undiluted allantoic fluid containing FluV, and added only the BC agent to the allantoic fluid containing NDV and IBV. The samples were adjusted at the same total volume for each virucidal agent using sterilized phosphate-buffered saline

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Table 1. Inactivation effects of virucidal agents against influenza virus (the adding method)

Virucidal agent	Virus strain	Final concentration of agent (%)	EID_{50}/0.2 mL at each treatment time						
			0 min[1)]	30 min	60 min	2 h	3 h	6 h	24 h
BC	A/Beijing/262/95	0.05	$10^{8.9}$	$\leqq 10^{1.1}$	<DL[2)]	<DL	NT	<DL	<DL
		0.1	$10^{8.9}$	<DL	<DL	<DL	NT	<DL	<DL
		0.2	$10^{8.9}$	<DL	<DL	<DL	NT	<DL	NT
	B/Guangdong/05/94	0.05	$10^{7.9}$	<DL	<DL	<DL	NT	<DL	<DL
		0.1	$10^{7.9}$	<DL	<DL	<DL	NT	<DL	<DL
		0.2	$10^{7.9}$	<DL	<DL	<DL	NT	<DL	NT
	NIBRG-14	0.1	$10^{7.8}$	<DL	NT	NT	<DL	NT	NT
SHC	A/Beijing/262/95	0.02	$10^{8.9}$	$10^{6.2}$	$10^{6.2}$	$10^{5.9}$	NT	$10^{6.3}$	$10^{6.1}$
		0.1	$10^{8.9}$	$10^{1.7}$	<DL	<DL	NT	NT	NT
		0.5	$10^{8.9}$	<DL	<DL	<DL	NT	NT	NT
	B/Guangdong/05/94	0.02	$10^{7.9}$	$10^{6.6}$	$\geqq 10^{6.6}$	$\geqq 10^{6.5}$	NT	$\geqq 10^{6.5}$	$\geqq 10^{6.1}$
		0.1	$10^{7.9}$	$10^{1.0}$	<DL	<DL	NT	NT	NT
		0.5	$10^{7.9}$	<DL	<DL	<DL	NT	NT	NT
SH	A/Beijing/262/95	0.5	$10^{8.9}$	$10^{2.5}$	$10^{3.4}$	$10^{2.6}$	NT	$\geqq 10^{4.3}$	$10^{2.9}$
		1	$10^{8.9}$	$10^{1.4}$	$10^{2.2}$	$\geqq 10^{2.3}$	NT	<DL	NT
	B/Guangdong/05/94	0.5	$10^{7.9}$	$10^{1.4}$	$10^{2.6}$	$10^{1.7}$	NT	<DL	<DL
		1	$10^{7.9}$	$10^{1.0}$	$10^{2.3}$	$10^{2.1}$	NT	<DL	NT
Negative control	A/Beijing/262/95	0	$10^{8.9}$	NT	NT	$10^{8.9}$	NT	$10^{9.3}$	NT
	B/Guangdong/05/94	0	$10^{7.9}$	NT	NT	$10^{8.7}$	NT	$10^{8.3}$	NT
	NIBRG-14	0	$10^{7.8}$	NT	NT	NT	$10^{8.2}$	NT	NT

[1)]: The indicated virus titers were derived from the data of the negative controls.
[2)]: <DL, less than detection limit (<$10^{1.0}$ EID_{50}/0.2 mL).
BC, benzalkonium chloride; SHC, sodium hypochlorite; SH, sodium hydroxide; NT, not tested.

Table 2. Inactivation effects of BC against Newcastle disease virus (the adding method)

Virucidal agent	Final concentration of agent (%)	EID_{50}/0.1 mL at each treatment time				
		0 min[1)]	30 min	60 min	2 h	24 h
BC	0.1	$10^{9.8}$	<DL[2)]	<DL	<DL	<DL
	0.2	$10^{9.8}$	<DL	<DL	<DL	<DL
Negative control	0	$10^{9.8}$	$\geqq 10^{10.5}$	$\geqq 10^{10.5}$	$\geqq 10^{10.5}$	$10^{9.7}$

[1)]: The indicated virus titers were derived from the data of the negative controls.
[2)]: <DL, less than detection limit (<$10^{1.1}$ EID_{50}/0.1 mL).
BC, benzalkonium chloride.

Table 3. Inactivation effects of BC against avian infectious bronchitis virus (the adding method)

Virucidal agent	Virus strain	Final concentration of agent (%)	EID_{50}/0.1 mL at each treatment time				
			0 min[1)]	30 min	60 min	2 h	24 h
BC	TM-86	0.1	$10^{6.1}$	<DL[2)]	<DL	<DL	<DL
		0.2	$10^{6.1}$	<DL	<DL	<DL	<DL
	Nerima	0.1	$10^{7.0}$	<DL	<DL	<DL	<DL
		0.2	$10^{7.0}$	<DL	<DL	<DL	<DL
Negative control	TM-86	0	$10^{6.1}$	$10^{5.5}$	$10^{6.0}$	$10^{6.1}$	$10^{5.8}$
	Nerima	0	$10^{7.0}$	$10^{6.5}$	$10^{6.3}$	$10^{6.3}$	$10^{6.1}$

[1)]: The indicated virus titers were derived from the data of the negative controls.
[2)]: <DL, less than detection limit (<$10^{0.9}$ EID_{50}/0.1 mL).
BC, benzalkonium chloride.

(PBS). The negative controls were prepared by adding only PBS to the allantoic fluids. All samples were incubated for several time periods at room temperature (RT) as shown in Tables 1-3. After incubation, 12% of sodium bisulfite (SB) was added to the samples containing SHC, and 90% of acetic acid was added to the samples containing SH for neutralization. All samples were stored at −80°C just after incubation until they were analyzed.

The wiping method: For this method, the B/Guangdong/05/94 strain of FluV was used. Four microliters of allantoic fluid containing the B/Guangdong/05/94 strain of FluV were deposited into each of four Petri dishes (diameter, 9.5 cm) for each virucidal agent experiment for BC, SHC, and ethanol. The allantoic fluid was spread to all areas of the Petri dish and the excess solution was removed. The allantoic fluid was then wiped up using cotton rectangles (6 × 5 cm) that each contained a different concentration of the virucidal agent as shown in Table 4. The negative controls were prepared by adding only PBS to the cotton. Then, 1 mL of sterilized PBS was applied to each wiped Petri dish, and the PBS was

Table 4. Inactivation effects of virucidal agents against influenza virus (B/Guangdong/05/94 strain) inactivation (the wiping method)

Virucidal agent	Agent concentration (%)	% of virus infected eggs
BC	0.05	0
	0.1	0
	0.2	0
SHC	0.02	100
	0.1	16.7
Ethanol	70	100
Negative control	0	100

BC, benzalkonium chloride; SHC, sodium hypochlorite.

recovered and stored at −80°C until analyzed. Neutralization of SHC was not performed using this method.

Estimation of virucidal activity for the adding method: Virucidal activity was estimated by measuring the virus infective titer of each sample. After thawing, the samples were serially diluted with sterilized PBS and were subjected to the following analyses (12,13):

For the residual FluV titration, 0.2 mL of each diluted sample was inoculated into the allantoic cavities of five eggs that were 11 days old for the A/Beijing/262/95 and B/Guangdong/05/94 strains and of five eggs that were 12 days old for the NIBRG-14 strain. Because the volume of the allantoic fluid per egg is 5 mL or more (14), the virucidal agent was diluted at least 25-fold just after inoculation. The inoculated eggs were incubated at 34°C for 2 days. The allantoic fluid was individually tested for agglutinability on an equal amount of 0.5% chicken red blood cell solution at RT for 60 min. Each 50% egg-infective dose (EID_{50}) per 0.2 mL was calculated using the Behrens-Kärber method.

For the residual NDV titration, 0.1 mL of each of the diluted samples was inoculated into the allantoic cavities of five eggs that were 10 days old. Because the volume of the allantoic fluid per egg is 5 mL or more (14), the virucidal agent was diluted at least 50-fold just after inoculation. The inoculated eggs were incubated at 37°C for 5 days. The allantoic fluid was individually tested for agglutinability on an equal amount of 0.5% chicken red blood cell solution at RT for 60 min. Each EID_{50}/0.1 mL was calculated using the Behrens-Kärber method.

For the residual IBV titration, 0.1 mL of each of the diluted samples was inoculated into the allantoic cavities of five eggs that were 9 days old. Because the volume of the allantoic fluid per egg is 5 mL or more (14), the virucidal agent was diluted at least 50-fold just after inoculation. The inoculated eggs were incubated at 37°C for 7 days. The allantoic fluid was individually tested where death or morphological changes of the avian embryo, appearing as dwarfing or curling into a spherical form (15), were a positive indication of infection. Each EID_{50}/0.1 mL was calculated using the Behrens-Kärber method.

When the EID_{50} was not within the performed sample dilutions, the EID_{50} was expressed using an inequality symbol.

Estimation of virucidal activity for the wiping method: Virucidal activity was estimated by measuring each sample's FluV infective percentage (12,16). After thawing, 0.2 mL of each sample was inoculated into the allantoic cavities of six eggs that were 11 days old. Because the volume of the allantoic fluid per egg is 5 mL or more (14), the virucidal agent was diluted at least 25-fold just after inoculation. The inoculated eggs were incubated at 34°C for 2 days. The allantoic fluid was individually tested for agglutinability on an equal amount of 0.5% chicken red blood cell solution at RT for 60 min. The positive percentage of each virus-infected egg was calculated.

RESULTS

Virucidal agents' effects on FluV inactivation by the adding method: The effects of BC, SHC, and SH in the adding method on the inactivation of the A/Beijing/262/95, B/Guangdong/05/94, and NIBRG-14 strains of FluV were investigated. The results are shown in Table 1. In the absence of a virucidal agent, both A/Beijing/262/95 and B/Guangdong/05/94 strains were stable even after incubation at RT for 6 h. The NIBRG-14 strain was stable for at least 3 h. The standard deviations (SDs) of EID_{50} (Log) and the coefficients of variations (CVs) of EID_{50} (Log) were 0.40 or less and 4.8% or less, respectively. The estimated detection limit (DL) was $10^{1.0}$ EID_{50}/0.2 mL due to the 2.58SD with a significance level of 1%. The A/Beijing/262/95 and B/Guangdong/05/94 strains were inactivated to less than the DL by the treatment with 0.05% of BC for 60 min. At 0.1 and 0.2% of BC, both the A/Beijing/262/95 and B/Guangdong/05/94 strains were inactivated to less than the DL with at least 30 min of treatment. Virus viability did not depend on the dose of BC. The NIBRG-14 strain at 0.1% BC was also inactivated to less than the DL with 30 min of treatment.

On the other hand, the treatment with 0.02% of SHC for 24 h did not inactivate to less than the DL either A/Beijing/262/95 or B/Guangdong/05/94. Moreover, the treatment with 0.1% of SHC for 30 min did not inactivate to less than the DL for either strain. Both strains were inactivated to less than the DL by the treatment with 0.5% of SHC for at least 30 min. Virus viability was dependent on the dose of SHC.

The treatment with 1% of SH for 2 h did not inactivate to less than the DL either A/Beijing/262/95 or B/Guangdong/05/94. Virus viability was not dependent on the dose of SH.

Effects of BC on NDV inactivation by the adding method: NDV in the absence of BC was stable even after incubation at RT for 24 h. The SD of EID_{50} (Log) and CV of EID_{50} (Log) were 0.41 and 4.0%, respectively. The estimated DL was $10^{1.1}$ EID_{50}/0.1 mL due to the 2.58SD, with a significance level of 1%. BC was effective for the inactivation of NDV. NDV was inactivated to less than the DL by the treatment with 0.1% of BC for 30 min, as shown in Table 2.

Effects of BC on IBV inactivation by the adding method: In the absence of BC, both TM-86 and Nerima strains were stable even after incubation at RT for 24 h. The SDs of EID_{50} (Log) and the CVs of EID_{50} (Log) were 0.34 or less and 5.3% or less, respectively. The estimated DL was $10^{0.9}$ EID_{50}/0.1 mL due to the 2.58SD, with a significance level of 1%. BC was also effective for the inactivation of the two strains of IBV. The treatment with 0.1% of BC for 30 min inactivated the IBV to less than the DL, as shown in Table 3.

Effects of several virucidal agents on FluV type B inactivation by the wiping method: In the case of 0.1% of BC, the complete inactivation was performed by the wiping method. On the other hand, at a comparable concentration (0.1%) of SHC, one of the six eggs was infected by viruses. Seventy percent of ethanol was not effective for the inactivation of the B/Guangdong/05/94 strain by this method. The data on the negative control showed that the wiping method did not affect the FluV titer. These results are shown in Table 4.

DISCUSSION

In this study, we investigated the effects of virucidal agents on the inactivation of FluV, NDV, and IBV using two methods (the adding and wiping), considering actual vaccine manufacturing conditions where it is needed for the efficient inactivation treatment of virus-contaminated waste and instruments.

Heating FluV in allantoic fluid at 100°C for 10 min inactivated both A/Beijing/262/95 and B/Guangdong/05/94 strains to less than the DL (data not shown). However, a major system, such as that using a large-scale autoclave, would be required to maintain heating at 100°C for a large amount of virus-contaminated allantoic fluid and a large number of virus-contaminated instruments in the vaccine manufacturing process. On the other hand, heating at 60°C, even for 6 h, did not achieve inactivation to less than the DL (data not shown). It can be said that FluV placed in the allantoic fluid of chicken eggs is stable, because it has been reported that heating at 56°C for 30 min can inactivate almost all viruses (17).

In general, BC is considered a less effective virucidal agent against most viruses (2,3,18). However, our results revealed that BC's virucidal activity against FluV, NDV, and IBV in allantoic fluid was more effective than those of SHC, SH, and ethanol. Since BC's virucidal activity was strong, virus viability was not dependent on the dose. This is because BC is a cationic soap. FluV, NDV, and IBV are viruses that have envelopes. Therefore, it was speculated that BC resolves the virus envelope as a detergent and that the activity may select negatively charged viruses because of electrostatic interaction. On the other hand, it can be considered that SHC, SH, and ethanol rely only on Brownian motion. The effective concentration of BC against all three viruses was similar. In addition, the Japanese encephalitis virus (JEV) also has single-stranded RNA with an envelope, and its vaccine is produced using mouse brain. BC's virucidal activity against JEV in the presence of a large amount of mouse brain proteins was comparable to that against FluV, NDV, and IBV in allantoic fluid (data not shown).

It is generally considered that the virucidal activities of SHC and SH against viruses are stronger than that of BC (18,19). However, in this study the reverse was true. SHC's virucidal activity is oxidization (19). Therefore, most of its activity may be inactivated by a high concentration of protein in allantoic fluid. Regarding SH, the virucidal activity is due to alkalization (19), and most of the activity may also be inactivated by a high concentration of protein in allantoic fluid.

The effective concentration of SHC was too high to be suitable for vaccine manufacturing. Its oxidation activity corrodes instruments (4). Also, neutralization is necessary using a neutralizing solution such as SB, which is harmful to humans, before the inactivated viral solution is discarded.

Based on our results, we think that BC is suitable and useful for the inactivation of FluV, NDV, IBV, and JEV in vaccine manufacturing, because its pH is almost neutral and its activity is easily inactivated by an ordinal negatively charged soap (4). Moreover, BC is not very harmful to humans (2,20-22) and is more easily biodegraded by activated sludge than SHC (23,24). BC's environmental effect is considered small if it is sufficiently diluted before disposal. Regarding the concentration that is effective for inactivation, 0.1% of BC was appropriate against FluV, NDV, and IBV. BC's virucidal activity on the inactivation of JEV was sufficient for vaccine manufacturing (data not shown) (25). Atomization of the 0.2% BC solution was also effective against FluV (data not shown). Also, new cell culture-derived vaccines have been in development (26-29). As the concentration of host cell protein is generally far lower than that of allantoic fluid and mouse brains, BC may also be useful for the cell culture-derived vaccine manufacturing process as a virucidal agent. In addition, because there was no difference in BC's inactivation effect among the A/Beijing/262/95, B/Guangdong/05/94, and NIBRG-14 strains of the FluV, it may also be effective against pandemic FluVs. Moreover, BC is commercially available and may contribute to public health.

In conclusion, BC is a useful virucidal agent, especially for FluV, NDV, and IBV vaccine manufacturing.

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