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—Original—

Effect of Weak Acid Hypochlorous Solution on Selected Viruses and Bacteria of Laboratory Rodents

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Abstract: Weak acid hypochlorous solution (WAHS) is known to have efficacy for inactivating pathogens and to be relatively safe with respect to the live body. Based on these advantages, many animal facilities have recently been introducing WAHS for daily cleaning of animal houses. In this study, we determined the effect of WAHS in inactivating specific pathogens of laboratory rodents and pathogens of opportunistic infection. WAHS with an actual chloride concentration of 60 ppm and a pH value of 6.0 was generated using purpose-built equipment. One volume of mouse hepatitis virus (MHV), Sendai virus, lymphocytic choriomeningitis virus, *Bordetella bronchiseptica*, *Pasteurella pneumotropica*, *Corynebacterium kutscheri*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* was mixed with 9 or 99 volumes of WAHS ($\times 10$ and $\times 100$ reaction) for various periods (0.5, 1, and 5 min) at 25°C. After incubation, the remaining infectious viruses and live bacteria were determined by plaque assay or culture. In the $\times 100$ reaction mixture, infectious viruses and live bacteria could not be detected for any of the pathogens examined even with the 0.5-min incubation. However, the effects for MHV, *B. bronchiseptica*, and *P. aeruginosa* were variable in the $\times 10$ reaction mixture with the 0.5- and 1-min incubations. Sufficient effects were obtained by elongation of the reaction time to 5 min. In the case of MHV, reducing organic substances in the virus stock resulted in the WAHS being completely effective. WAHS is recommended for daily cleaning in animal facilities but should be used properly in order to obtain a sufficient effect, which includes such things as using a large enough volume to reduce effects of organic substances.

Key words: antiviral effect, bactericidal effect, opportunistic pathogens, specific pathogens, weak acid hypochlorous solution

Introduction

In order to obtain consistent and reliable results from animal experiments, laboratory rodents should be free from infectious microbes that affect various biological functions. Animal facilities are constantly trying to prevent these kinds of infections in laboratory animals. Regardless of their efforts, microbial outbreaks have been still reported at many animal facilities [13, 14, 18, 20, 21].

Cleaning of the animal house environment using disinfectants is one of the most important procedures in animal facilities. Iodine-, chlorine-, and alcohol-based disinfectants and quaternary ammonium compounds are mainly used for daily hygiene management. Each disinfectant has its own merits and demerits. Iodine-based disinfectants have strong biocidal activity but have the disadvantage of being highly corrosive. Alcohol-based disinfectants require careful handling because of their

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Table 1. Microorganisms and their strains used in this study

	Pathogen	Strain	Proliferation
Virus	Mouse hepatitis virus	JHM	DBT cell
	Sendai virus	Z	egg allantoic cavity
	Lymphocytic choriomeningitis virus	Armstrong	Vero E6 cell
Bacteria	<i>Bordetella bronchiseptica</i>	R5	DHL agar
	<i>Pasteurella pneumotropica</i>	MaM	Blood agar
	<i>Corynebacterium kutscheri</i>	S5L	FNC agar
	<i>Staphylococcus aureus</i>	SL-1	Mannitol salt agar
	<i>Pseudomonas aeruginosa</i>	YoM-C	NAC agar

irritating and flammable properties. Chlorine-based disinfectants have strong biocidal activity and are therefore widely used in food preparation, medical facilities, and care homes. However, chlorine-based disinfectants have disadvantages of corrosiveness to metals, being highly irritating, and having a strong odor. Quaternary ammonium compounds have less harmful properties, but are not effective for some kinds of viruses and bacteria [2, 4, 7, 8, 11].

In chlorine-based disinfectants, free chlorine exists in mainly two different forms, hypochlorous acid (HOCl) and hypochlorite ion (OCl⁻) [5]. HOCl is much more effective than OCl⁻ as a disinfectant, and the pH of the solution affects the proportions of these two forms [10]. Weak acid hypochlorous solution (WAHS) is a chlorine-based disinfectant that is produced by mixing NaClO and HCl in water and adjusting it to a weak acidity of around pH 6 using a purpose-built equipment. The main free chlorine in WAHS is HOCl, the more effective form, because of its weak acidity. WAHS is biologically safe and less irritating, although it remains somewhat corrosive. WAHS has been reported to have an excellent microbicidal effect against a broad range of microorganisms related to human health, so WAHS has practical applicability in hospitals or the food industry [12].

Recently, many animal facilities have introduced WAHS for maintaining a clean environment in facilities in Japan. Its effect on laboratory animal-specific microorganisms, however, has not been reported. Recently, we reported that WAHS inactivated mouse norovirus [17].

In this study, we investigated the effect of WAHS on selected viruses and bacteria of laboratory rodents, i.e., those that are included in the microbiological monitoring menu of normal animals and cause opportunistic infections with pathogenicity in only immunodeficient animals.

Materials and Methods

Disinfectant preparation

WAHS was prepared with a pH of 6.0 ± 2.0 and an available chlorine concentration of 60 ppm by mixing 6% sodium hypochlorite (HSP soda, HSP Co., Ltd., Okayama, Japan), 8.5% hydrochloric acid (HSP acid, HSP Co., Ltd.), and tap water in a WAHS production device (HSP-SR600 Steri Revo, HSP Co., Ltd.). WAHS was used immediately after output from a tap of the production device without dilution. A pH meter (MP230, Mettler-Toledo International Inc., Tokyo, Japan) was used to measure pH, and a high-concentration residual chlorine meter (RC-7Z, Kasahara Chemical Instruments Co., Ltd., Tokyo, Japan) was used to measure the available chlorine concentration in WAHS. A chlorine-based disinfectant was prepared by diluting 6% sodium hypochlorite (Purelox, OYALOX Co., Ltd., Tokyo, Japan) with sterilized water to achieve a concentration of 0.03% ($\times 200$ dilution). An alcohol-based disinfectant was prepared by mixing 99.9% ethanol with sterilized water at a ratio of 7:3 to achieve a final concentration of 70%. The sodium hypochlorite and ethanol disinfectants were used for experiments immediately after preparation.

Preparation of viral solutions

The antiviral effects on mouse hepatitis virus (MHV), Sendai virus (HVJ), and lymphocytic choriomeningitis virus (LCMV) were determined. The strains of viruses used in this study are shown in Table 1. MHV was proliferated in DBT cells in Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich Inc., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) and 10% tryptose phosphate broth (TPB) [6]. LCMV was proliferated in Vero E6 cells in Dulbecco's Modified Essential Medium (DMEM) (Sigma-Aldrich Inc.) containing 10% FBS [16]. HVJ was cultured in the egg allantoic cavity

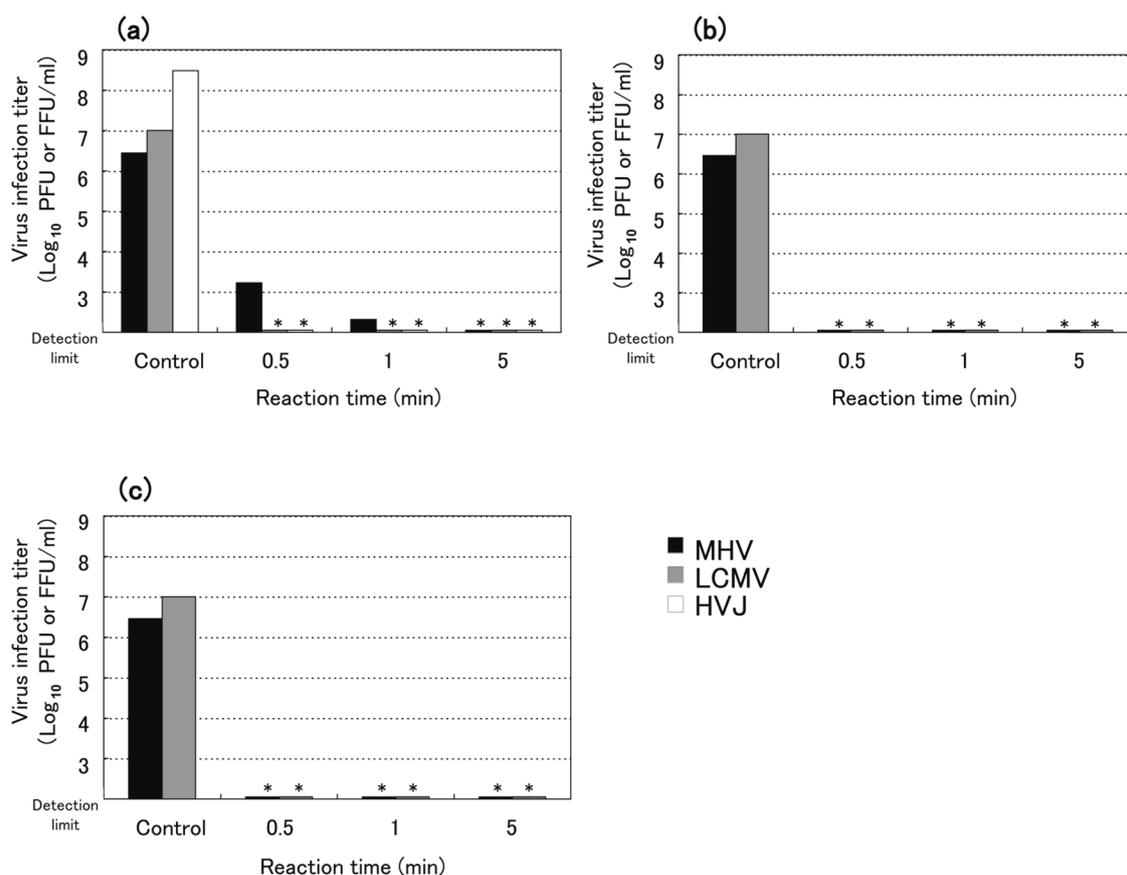


Fig. 1. Antiviral effects of disinfectants in the $\times 10$ reaction mixture. (a) Effect of WAHS. For MHV, the results of all reaction times are shown as means of 7 experiments. (b) Effect of 0.03% sodium hypochlorite. (c) Effect of 70% ethanol. As a control, bacterial solutions were mixed with the final $\times 10$ sterilized PBS and reacted for 5 min at 25°C. The culture medium for the MHV stock was MEM containing 10% FBS and 10% TPB. After reaction, infectious viruses were titrated in culture cells. The effect on HVJ was determined only using WAHS. The vertical bars show the viable bacteria counts, and 2.0 log₁₀ represents the detection limit. Asterisks indicate values that are less than the detection limit.

[3, 15]. After a proper incubation period for proliferation of each virus, the culture medium or allantoic fluid was centrifuged at 3,000 rpm for 5 min at 4°C, and the supernatant was used as the virus solution. The titers of virus stocks were assayed by methods previously reported in plaque forming units (PFU)/ml for MHV in DBT cells and HVJ in CV-1 cells in MEM containing 1% FBS and 0.25% trypsin and in focus forming units (FFU)/ml for LCMV in Vero E6 cells [19]. In the experiment concerning reduction of the FBS concentration, MHV was proliferated in MEM containing 2% FBS without TPB. Virus stocks were frozen and stored at -80°C until use. Viral solutions for mixing with disinfectants were adjusted to 10⁶–10⁸ PFU/ml or FFU/ml.

Preparation of bacterial solutions

The bactericidal effects on *Bordetella bronchiseptica*, *Pasteurella pneumotropica*, *Corynebacterium kutscheri*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were determined. The strains of bacteria used in this study are shown in Table 1. Agars were prepared in our lab using powder products of Eiken Chemical Co., Ltd., Tokyo, Japan. *B. bronchiseptica* on DHL agar, *P. pneumotropica* on blood agar [9], *C. kutscheri* on FNC agar [1], *S. aureus* on mannitol salt agar, and *P. aeruginosa* on NAC agar were cultured for 24 h at 37°C. Colonies were harvested from plates and suspended in phosphate-buffered saline (PBS). The actual bacteria concentration of a prepared bacteria suspension was determined by plating 1 ml of serially diluted suspension, spreading on the surface of an agar with a spreader until almost no

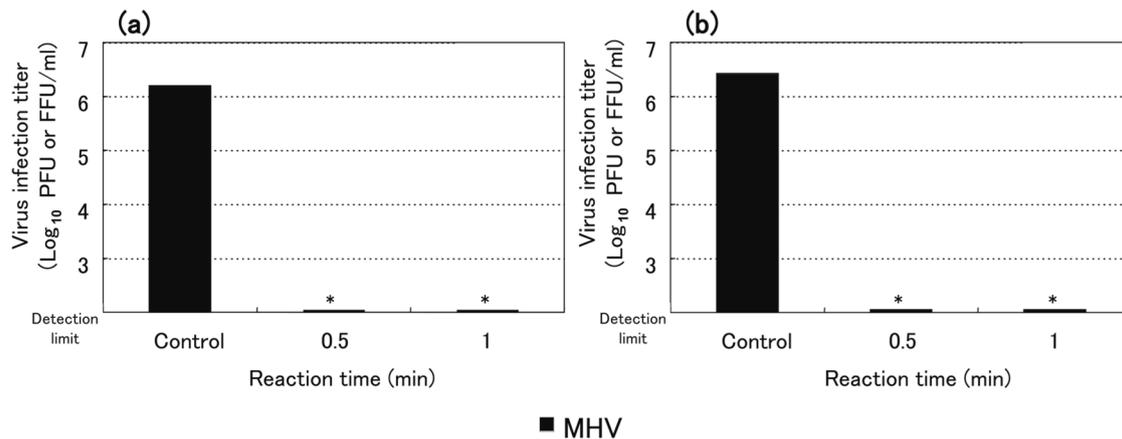


Fig. 2. Effect of medium conditions for MHV viral stock and reaction ratio on the efficacy of WAHS. (a) The culture medium for MHV stock was changed to MEM containing 2% FBS without TPB. (b) Viral solution was mixed with the final $\times 100$ volume of WAHS. Asterisks indicate values that are less than the detection limit.

liquid remained, and incubation for 24 h at 37°C. Then, viable bacteria were counted. Bacterial solutions used in this paper contained 10^6 – 10^9 colony forming units (CFU)/ml.

Measurement of inactivating effect on viruses

One hundred or 10 μ l of viral solutions were mixed with 900 or 990 μ l of disinfectants, a $\times 10$ or $\times 100$ reaction, respectively, and reacted for 3 time periods (0.5, 1, and 5 min) at 25°C. Experiments were performed in triplicate except where specifically mentioned. As a control, viral solutions were mixed with sterilized PBS and reacted for 5 min at 25°C. The remaining infectious virus was titrated by PFU assay for MHV and HVJ and by FFU assay for LCMV.

Measurement of inactivating effect on bacteria

One hundred or 10 μ l of bacterial solutions were mixed with 900 or 990 μ l of disinfectants, a $\times 10$ or $\times 100$ reaction, respectively, and reacted for 3 time periods (0.5, 1, and 5 min) at 25°C. At the end of the incubation period, 1 ml of heart infusion broth was added to the solution. Experiments were performed in triplicate except where specifically mentioned. As a control, bacterial solutions were mixed with sterilized physiological saline and reacted for 5 min at 25°C. One milliliter of reaction mixture was cultured on agars for 24 h at 37°C, and viable bacteria were counted.

Results

Antiviral effects

WAHS showed highly antiviral effects on LCMV and HVJ even in 0.5 min, since the infectious virus titer was below the detection limit when viral solutions were mixed with $\times 10$ WAHS (Fig. 1a.). However, the effect on MHV showed some variation. A complete antiviral effect of WAHS on MHV was obtained in just 1 out of 7 experiments after 0.5 and 1 min, respectively. Even in the case that infectious virus was detected, the virus titers were reduced by more than 3 to 4 log₁₀ compared with the control. A complete antiviral effect on MHV was obtained after a reaction time of 5 min with the $\times 10$ reaction mixture. The results for MHV in Fig. 1a show means of infectious virus titers of the 7 experiments for all reaction times. Sodium hypochlorite and ethanol showed high inactivating effects on MHV and LCMV with the $\times 10$ reaction mixture (Fig. 1b and 1c).

Adequate efficacy was also obtained when MHV virus stock was prepared in the medium containing 2% FBS without TPB and mixed with WAHS with the $\times 10$ reaction mixture (Fig. 2a). A complete antiviral effect was also obtained when the mixture ratio was changed to $\times 100$ (Fig. 2b).

Antibacterial effects

All 3 disinfectants, WAHS, sodium hypochlorite, and ethanol, showed highly antibacterial effects on *P. pneumotropica*, *C. kutscheri*, and *S. aureus* even after reaction for 0.5 min in the $\times 10$ reaction mixture, since no viable

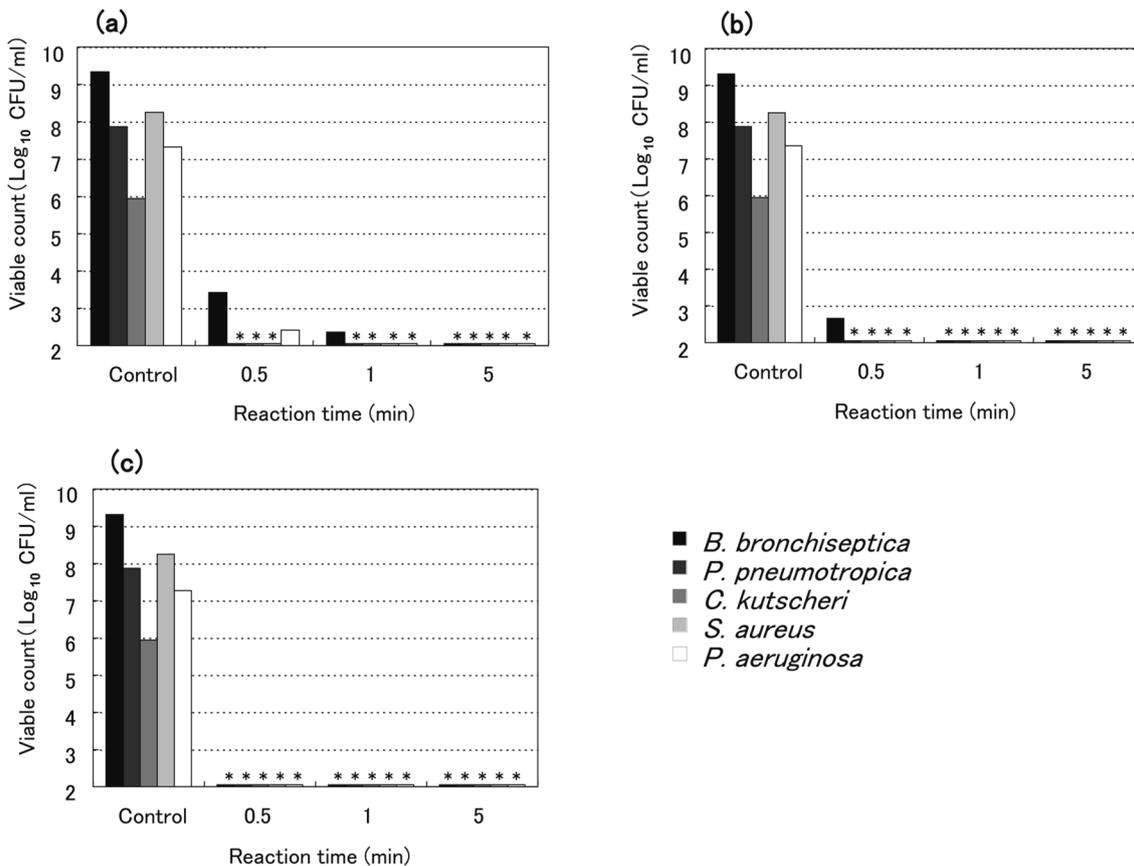


Fig. 3. Antibacterial effects of disinfectants in the $\times 10$ reaction mixture. (a) Effect of WAHS. For *B. bronchiseptica* and *P. aeruginosa*, the results for 0.5 and 1 min are shown as means of 6 experiments. (b) 0.03% sodium hypochlorite. For *B. bronchiseptica*, the results for 0.5 min are shown as the mean of 5 experiments. (c) Effect of 70% ethanol. As a control, bacterial solutions were mixed with the final $\times 10$ sterilized physiological saline and reacted for 5 min at 25°C. After reaction, viable bacteria were cultured on agars. The vertical bars show viable bacteria counts, and 2.0 log₁₀ represents the detection limit. Asterisks indicate values that are less than the detection limit.

bacteria were evident (Fig. 3). However, there was some variation in inactivating effects of WAHS on *B. bronchiseptica* and *P. aeruginosa* in the $\times 10$ reaction mixture (Fig. 3a). A complete antibacterial effect of WAHS on *B. bronchiseptica* was obtained in 2 out of 6 experiments after 0.5 min and in 4 out of 6 experiments after 1 min. A complete inactivation effect was observed with elongation of the reaction time to 5 min. Viable *P. aeruginosa* was detected after a 0.5-min reaction in 2 out of 6 experiments even though the remaining viable bacteria had very low titers. The results in Fig. 3a show means of viable bacteria counts of the 6 experiments for 0.5 and 1 min. Even in the case that a complete effect was not observed, the titers decreased by more than 4 to 5 log₁₀ compared with the control in each experiment. The effects of sodium hypochlorite on *B. bronchiseptica* were also variable in the $\times 10$ reaction mixture (Fig. 3b). Viable

bacteria were detected in 2 out of 5 experiments after 0.5 min, and Fig. 3b shows the mean viable bacteria counts of the 5 experiments. After reaction for 1 and 5 min, no viable *B. bronchiseptica* was detected.

We then changed the volume ratio of the disinfectants to be mixed with bacterial solution. Fig. 4 shows the results of the $\times 100$ reaction mixture. *B. bronchiseptica* and *P. aeruginosa* were completely inactivated with WAHS and sodium hypochlorite even after reaction for 0.5 min.

Discussion

The antiviral and antibacterial activity of WAHS was investigated in this study with respect to laboratory rodent-specific and opportunistic pathogens and compared with two other well-used disinfectants, sodium hypo-

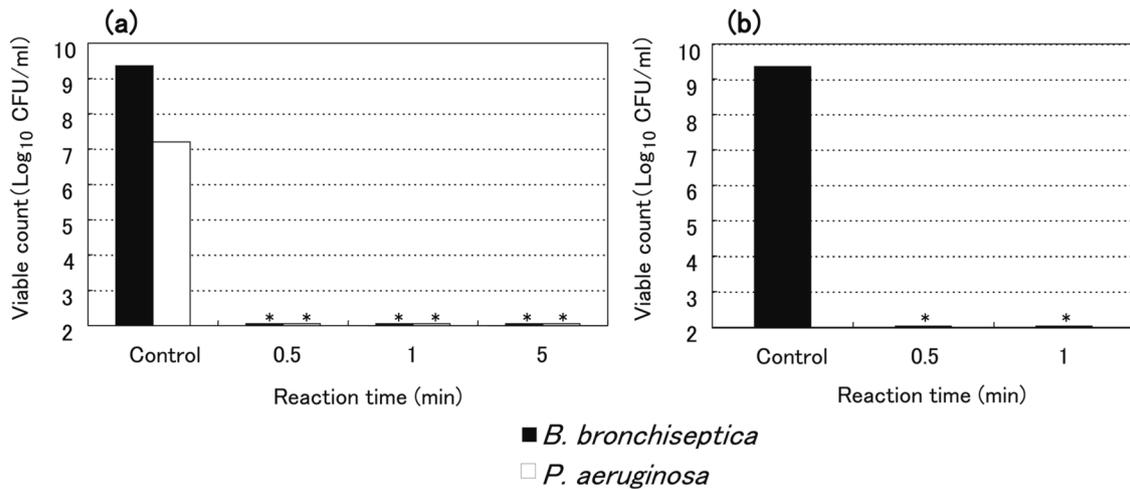


Fig. 4. Antibacterial effect of disinfectants in the $\times 100$ reaction mixture. (a) Effect of WAHS. (b) Effect of 0.03% sodium hypochlorite. As controls, bacterial solutions were mixed with the final $\times 100$ sterilized physiological saline and reacted for 5 min at 25°C. After reaction, viable bacteria were cultured on agars. The vertical bars show viable bacteria counts, and 2.0 log₁₀ represents the detection limit. Asterisks indicate values that are less than the detection limit.

chlorite and ethanol. WAHS was adequately effective within a short reaction time when mixed with pathogens at a sufficient volume ratio ($\times 100$). However, the activity was sometimes reduced when WAHS was mixed at a low volume ratio ($\times 10$). Reduction of activity was also observed in the case of sodium hypochlorite. Both WAHS and sodium hypochlorite showed sufficient activity with a longer reaction time. Ethanol was very effective at both ratios and all reaction times.

The main effective form of chlorine in WAHS is HOCl. HOCl oxidizes organic materials that cause a reduction in biocidal effect [5]. There is a possibility that an organic substance included in the MHV virus stock, such as FBS and TPB, interfered with the effect of WAHS, since the MHV virus stock was usually proliferated in a culture medium containing 10% FBS and 10% TPB. This hypothesis was confirmed, since sufficient antiviral activity was obtained when the concentration of FBS in the MHV viral stock was reduced to 2% and TPB was not added to the medium.

Although WAHS efficacy was affected by the probable presence of organic substances, WAHS had sufficient inactivating activity against laboratory rodent-specific viruses and bacteria tested when used with a sufficient volume or longer reaction time. Sodium hypochlorite and ethanol are very effective disinfectants, but they have demerits such as being highly corrosive to metals,

having strong odors, being irritants, and being flammable. WAHS still has the demerits of corrosiveness and odor, but these properties are very weak compared with those of sodium hypochlorite. The big merit of WAHS is that it can be used without dilution. Therefore, WAHS is recommended for daily use in cleaning animal facilities under proper usage conditions, such as use of a larger volume of WAHS or reducing organic substances.

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