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

Motoko TAHARAGUCHI, Kazuhiro TAKIMOTO, Aya ZAMOTO-NIIKURA, and Yasuko K. YAMADA

Division of Experimental Animal Research, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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 (×10 and ×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 ×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 ×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...
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% (×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.

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

**Table 1. Microorganisms and their strains used in this study**
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 $10^6$–$10^8$ 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 colonies were visible.
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 $10 \mu l$ of viral solutions were mixed with 900 or 990 $\mu l$ 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. 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).

**Measurement of inactivating effect on bacteria**

One hundred or $10 \mu l$ of bacterial solutions were mixed with 900 or 990 $\mu l$ 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 log$_{10}$ 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. 2a and 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
bacteria were evident (Fig. 3). However, there was some variation in inactivating effects of WAHS on *B. bronchiseptica* and *P. aeruginosa* in the ×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 log_{10} compared with the control in each experiment. The effects of sodium hypochlorite on *B. bronchiseptica* were also variable in the ×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 ×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 hypochlorite.
chlorite and ethanol. WAHS was adequately effective within a short reaction time when mixed with pathogens at a sufficient volume ratio (×100). However, the activity was sometimes reduced when WAHS was mixed at a low volume ratio (×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.

Fig. 4. Antibacterial effect of disinfectants in the ×100 reaction mixture. (a) Effect of WAHS. (b) Effect of 0.03% sodium hypochlorite. As controls, bacterial solutions were mixed with the final ×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 log_{10} represents the detection limit. Asterisks indicate values that are less than the detection limit.

References

1. Amao, H., Saito, M., Takahashi, K.W., and Nakagawa, M. 1990. Selective medium for Corynebacterium kutscheri and localization of the organism in mice and rats. Jikken Dobutsu 39: 519–529 (in Japanese with English abstract). [Medline]
2. Belliot, G., Lavaux, A., Souihel, D., Agnello, D., and Pothier, P. 2008. Use of Murine Norovirus as a Surrogate To Evaluate Resistance of Human Norovirus to Disinfectants. Appl. Environ. Microbiol. 74: 3315–3318. [Medline] [CrossRef]
3. Darlington, R.W., Portner, A., and Kingsbury, D.W. 1970. Sendai virus replication: an ultrastructural comparison of productive and abortive infections in avian cells. J. Gen. Virol. 9: 169–177. [Medline] [CrossRef]
4. Engelbrecht, K., Ambrose, D., Sifuentes, L., Gerba, C., Weart, I., and Koenig, D. 2013. Decreased activity of commercially available disinfectants containing quaternary ammonium compounds when exposed to cotton towels. Am. J. Infect. Control 41: 908–911. [Medline] [CrossRef]
5. Fukuzaki, S. 2006. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol.
6. Hirano, N., Murakami, T., Fujiwara, K., and Matsumoto, M. 1978. Utility of mouse cell line DBT for propagation and assay of mouse hepatitis virus. *Jpn. J. Exp. Med.* 48: 71–75. [Medline] [CrossRef]

7. Hirayama, M. 2011. The antimicrobial activity, hydrophobicity and toxicity of sulfonium compounds, and their relationship. *Biocontrol. Sci.* 16: 23–31. [Medline] [CrossRef]

8. Kawana, R., Kitamura, T., Nakagomi, O., Matsumoto, I., Arita, M., Yoshihara, N., Yanagi, K., Yamada, A., Morita, O., Yoshida, Y., Furuya, Y., and Chiba, S. 1997. Inactivation of human viruses by povidone-iodine in comparison with other antiseptics. *Dermatology 195 (Suppl 2):* 29–35. [Medline] [CrossRef]

9. Knight, D.P., Paine, J.E., and Speller, D.C. 1983. A selective medium for *Pasteurella multocida* and its use with animal and human specimens. *J. Clin. Pathol.* 36: 591–594. [Medline] [CrossRef]

10. Le Dantec, C., Duguet, J.P., Montiel, A., Dumoutier, N., Dubrou, S., and Vincent, V. 2002. Chlorine disinfection of atypical mucobacteria isolated from a water distribution system. *Appl. Environ. Microbiol.* 68: 1025–1032. [Medline] [CrossRef]

11. Matsuhira, T., Kaji, C., Murakami, S., Maebashi, K., Oka, T., Takeda, N., and Katayama, K. 2012. Evaluation of four antiseptics using a novel murine norovirus. *Exp. Anim.* 61: 35–40. [Medline] [CrossRef]

12. Ono, T., Yamashita, K., Murayama, T., and Sato, T. 2012. Microbicidal effect of weak acid hypochlorous solution on various microorganisms. *Biocontrol. Sci.* 17: 129–133. [Medline] [CrossRef]

13. Pritchett-Corning, K.R., Cosentino, J., and Clifford, C.B. 2009. Contemporary prevalence of infectious agents in laboratory mice and rats. *Lab. Anim.* 43: 165–173. [Medline] [CrossRef]

14. Schoondermark-van de Ven, E.M.E., Philipse-Bergmann, I.M.A., and van der Logt, J.T.M. 2006. Prevalence of naturally occurring viral infections, *Mycoplasma pulmonis* and *Clostridium piliforme* in laboratory rodents in Western Europe screened from 2000 to 2003. *Lab. Anim.* 40: 137–143. [Medline] [CrossRef]

15. Sugita, K., Maru, M., and Sato, K. 1974. A sensitive plaque assay for Sendai virus in an established line of monkey kidney cells. *Jpn. J. Microbiol.* 18: 262–264. [Medline] [CrossRef]

16. Takimoto, K., Taharaguchi, M., Morikawa, S., Ike, F., and Yamada, Y.K. 2008. Detection of the antibody to lymphocytic choriomeningitis virus in sera of laboratory rodents infected with viruses of laboratory and newly isolated strains by ELISA using purified recombinant nucleoprotein. *Exp. Anim.* 57: 357–365. [Medline] [CrossRef]

17. Takimoto, K., Taharaguchi, M., Sakai, K., Takagi, H., and Yamada, Y.K. 2013. Attempt to eliminate murine norovirus from mice and to prevent mice from murine norovirus infection by drinking of hypochlorite-based disinfectants. *Exp. Anim.* 62: 237–245. [Medline] [CrossRef]

18. Takimoto, K., Yamada, Y.K., Ami, Y., Suzuki, Y., Yabe, M., and Asano, T. 1999. Experiences of microbial contamination of animal colonies maintained in the National Institute of Infectious Diseases, Japan (NIID). *Jpn. J. Infect. Dis.* 52: 255–256. [Medline]

19. Tanishita, O., Takahashi, Y., Okuno, Y., Yamashita, K., and Takahashi, M. 1984. Evaluation of focus reduction neutralization test with peroxidase-antiperoxidase staining technique for hemorrhagic fever with renal syndrome virus. *J. Clin. Microbiol.* 20: 1213–1215. [Medline]

20. Urano, T., Noguchi, K., Jiang, G., and Tsukumi, K. 1995. Survey of *Pseudomonas aeruginosa* contamination in human beings and laboratory animals. *Exp. Anim.* 44: 233–239. [Medline] [CrossRef]

21. Yamamoto, H., Sato, H., Yagami, K., Arikawa, J., Furuya, M., Kurosawa, T., Mannen, K., Matsubayashi, K., Nishimune, Y., Shibahara, T., Ueda, T., and Itoh, T. 2001. Microbiological contamination in genetically modified animals and proposals for a microbiological test standard for national universities in Japan. *Exp. Anim.* 50: 397–407. [Medline] [CrossRef]