Biocontrol Science, 2012, Vol. 17, No. 3, 129−133

Note

Microbicidal Effect of Weak Acid Hypochlorous Solution on Various Microorganisms

TOMOKO ONO[1]*, KOJI YAMASHITA[1], TOMOMI MURAYAMA[2], AND TOSHIO SATO[2]

[1]*Research and Technology Development Division, HSP company, 56-5, Ezaki, Naka-ku, Okayama-shi, Okayama 702-8005, Japan*
[2]*Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu, Matsue-shi, Shimane 690-8504, Japan*

Received 10 June, 2011/Accepted 3 March, 2012

We investigated the microbicidal effect of weak acid hypochlorous solutions of pH 5.0 - 6.0, produced by mixing NaClO and HCl in water, against various bacteria, fungi, and virus *in vitro.* The weak acid hypochlorous solution had excellent microbicidal effect against a broad microbicidal spectrum of standard strains and clinical isolates in a short time. The microbicidal effects of hypochlorous solutions did not depend on the available chlorine concentration but on the HClO concentration. These results show that the weak acid hypochlorous solution has practical applicability in such places as hospitals and establishments related to the food industry.

Key words : Weak acid hypochlorous solution/Microbicidal effect/Standard strains/Clinical isolates.

In recent years, food poisoning caused by microorganisms such as *Salmonella* or *Norovirus* has become a serious problem in the food industry in which food products are mass-manufactured (Hayashi, 2010; Shinagawa, 2010). Hospital infections due to drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multi-drug resistant *Acinetobacter baumannii* (MRAB), and spore-forming bacteria such as *Bacillus cereus* and *Clostridium difficile* have increased in hospitals and nursing homes (Weber et al., 2010; Tomono, 2010). To control such harmful microorganisms, sodium hypochlorite (NaClO) is a versatile disinfectant, that is very useful because it is highly microbicidal, safe (WHO, 2008), and affordable. On the other hand, it has such demerits as being highly corrosive of metals, forming trihalomethane and being difficult to dilute for practical use (Ono, 2009). The aqueous solution of sodium hypochlorite is alkaline, and free available chorine exists as hypochlorite ion (OCl^-). It is known that the chlorine form is changed to undissociated hypochlorous acid (HClO) by controlling the pH values of the solution (Nakagawa et al. 1998), and HClO is a more effective disinfectant than ClO^- (Le Dantec, at al., 2002; Leyer, and Johnson, 1997; Leahy et al., 1987; Wang et al., 2007). Some reports refer to the microbicidal effect of a weak acid hypochlorous solution of pH 5.5-6.5 produced by mixing NaClO and HCl in water and see it as a promising disinfectant solution (Ono et al. 2006a, 2006b, and 2010).

The purpose of this study is to investigate the microbicidal effect of the weak acid hypochlorous solution against bacteria, virus, and fungi. We used 57 bacterial strains (10 standard strains and 47 clinical isolates), two standard fungal strains and a standard virus strain in this test. Standard strains and clinical isolates are shown in Table 1 and Table 2, respectively.

Fifty ppm hypochlorous solution was prepared by diluting 12% sodium hypochlorite with tap water. The pH value of the solution was adjusted to pH 5, 6, 7 or 8 with HCl or NaOH. The available chlorine

* *Corresponding author. Tel: +81-86-277-0888, Fax: +81-86-274-0839, E-mail: ono(a)hsp-net.co.jp*

TABLE 1. Standard strains.

Standard strains		
Bacteria	Gram negative	*Pseudomonas aeruginosa* ATCC27853 *Escherichia coli* ATCC25992 *Stenotrophomonas maltophilia* ATCC13637 *Acinetobacter baumannii* JCM6841 *Salmonella* Typhimurium IFO12529
	Gram positive	*Staphylococcus aureus* ATCC25923 *Enterococcus faecalis* ATCC29212 *Enterococcus feacium* ATCC35667 *Bacillus subtilis* ATCC6633 (spore) *Bacillus cereus* NBBC13597(spore)
Fungi		*Candida albicans* ATCC10231 *Aspergillus niger* IFO4407
Bacteriophage		*Phage* Qβ

TABLE 2. Clinical isolates.

Clinical isolate strains		No. of strains
Bacteria	*Pseudomonas aeruginosa*	10
	Acinetobacter baumannii	1
	Staphylococcus aureus (MRSA)	5
	Staphylococcus aureus (MSSA)	6
	Enterococcus faecalis	8
	Enterococcus feacium	2
	Enterococcus avium	1
Fungi	*Candida albicans*	10
	Candida grabrata	2
	Candida krusei	1
	Candida tropicalis	1

concentration was measured with the residual chlorine checker (the Pocketable Water Quality Photometer AQ-102 SIBATA SCIENTIFIC TECHNOLOGY LTD., Saitama, Japan).

Bacterial strains were inoculated upon standard method agar plates (SDA Niisui, Tokyo, Japan) for 24 h at 37℃. Fungal strains were inoculated and grown on Sabouraud agar (Nissui, Tokyo, Japan) for 7 d. After incubation, the colonies were picked up and suspended in sterilized distilled water. Spore-forming bacteria (*B. subtilis* and *B. cereus*) grown on SDA for 14 d were suspended in sterilized normal saline solutions. The spore suspensions were heated at 60℃ for 1 min to kill their vegetative cells, and washed twice with normal saline solution using centrifugation. Before the bactericidal test, the existence of the spores was confirmed by the microscopy. Phage Qβ was incubated in liquid medium for propagation with the cells of *E. coli* (*Escherichia coli* K-12F$^+$ A/λ) as the host strain at 37℃ (Sunayama et al., 2002). After incubation, the culture broth was centrifuged (4℃, 14,310×g, 20min) and filtered with a 0.45 μm membrane filter (Cat.No.A045A047A., Advantec Toyo Kaisya Ltd., Tokyo, Japan) to remove the host strain.

To the 0.2ml of bacterial or fungal suspension was added 1.8ml of 50ppm hypochlorous solution, and mixing was done by a Boltex mixer under room temperature. 4 ml of the brain heart infusion broth (BHI broth Japan, Becton, Dickinson and Company, Tokyo, Japan) was added to the bacterial or fungal suspensions at predetermined regular time intervals to inactivate the available chlorine. The BHI broths were incubated for 72 h at 37℃, and bacterial growth effect was assessed by their increase in turbidity. In the case of *Aspergillus niger*, a 0.1 ml of the suspension after incubation with hypochlorous solution was smeared on the potato dextrose agar (PDA Nissui, Tokyo, Japan). It was incubated for 14 d at 24 ℃ and the colonies that appeared on the plate were counted. In the case of phage Qβ, 0.1 ml of the suspension after incubation with hypochlorous solutions and *E. coli* cell culture medium was smeared on the culture media for enumeration of the bacteriophage. The plaques that appeared on the plate were counted by the plaque forming unit method.

Table 3 shows the microbicidal effects of hypochlorous solutions against the standard strains. The values are represented as the minimum contact time to give culture-negative results. Using hypochlorous solutions with 50ppm available chlorine, all kinds of the standard strains were killed with a contact time of 10 to 3,600 s. Especially, *Pseudomonas aeruginosa*, *E. coli*, *A. baumannii*, *S. aureus*, *Enterococcus faecalis*, *E. faecium*, and phage Qβ became culture-negative within 15 s in the pH range of 5-8. On the other hand, *B. subtilis*, *B. cereus, S. maltophilia*, and *A. niger* required longer sterilizing times than the aforementioned bacteria. For instance, *B. subtilis* became culture-negative at 3,600 s under the pH 8 conditions, but at only 300 s under the pH 5 and 6 conditions.

Table 4 shows the microbicidal effects of hypochlorous solutions against the clinical isolates at 15 s. All strains of bacteria became culture-negative at 15 s. All strains of fungi became negative at 15 s in the pH range from 5 to 7. However, under conditions at pH 8, some fungi strains were culture-positive after 15 s.

These results indicated that the bactericidal effects are higher under the weak acidic pH conditions (pH : 5.0-6.0). It is due to the fact that most of the hypochlorite ions (ClO^-) in the alkaline solution are changed to hypochlorous acid (HClO) by adjusting the pH to the weak acid region. The bactericidal effect of HClO is closely connected with the membrane permeability of bacteria cells (Fukuzaki, 2006; Urano and Fukuzaki, 2005). Ionized ClO^- is hardly able to penetrate the bacterial cell membranes with a

TABLE 3. Microbicidal effect of hypochlorous solutions against standard strains.

Standard strains	Control (CFU/ml)	pH 5	pH 6	pH 7	pH 8
		First negative (sec)			
P.aeruginosa ATCC27853	1.1-16 × 10^7	15	15	15	15
E.coli ATCC25992	2.0-9.2 × 10^7	15	15	15	15
S.maltophilia ATCC13637	2.1-7.0 × 10^7	60	60	180	180
A.baumannii JCM6841	1.7-5.3 × 10^7	15	15	15	30
*S.*Typhimurium IFO12529	3.8-6.9 × 10^7	15	30	30	30
S.aureus ATCC25923	2.0-16 × 10^7	15	15	15	15
E.faecalis ATCC29212	1.1-11 × 10^7	15	15	15	15
E.faecium ATCC35667	1.4-16 × 10^7	15	15	15	15
B.subtilis ATCC6633 (spore)	4.7-7.2 × 10^7	300	300	600	3600
B.cereus NBBC13597 (spore)	3.7-7.1 × 10^7	600	600	1800	3600
Candida albicans ATCC10231	1.0-3.4 × 10^7	15	15	15	30
Aspergillus niger IFO4407	1.8-5.5 × 10^6	300	300	900	1800
Phage Q β	1.0 × 10^6	15	15	15	15

The available chlorine concentration was 50ppm
Detection limit was 0.5 CFU/ml. (In case of *A. niger* and Phage *Q β*, detection limit was 10 CFU/ml)

TABLE 4. The microbicidal effects against clinical isolates.

	Clinical isolates	No. of strains	Control (CFU/ml)	pH 5	pH 6	pH 7	pH 8
				Positive number of strains / Total number of strains			
Bacteria	*P. aeruginosa*	10	1.1-16 × 10^7	0/10	0/10	0/10	0/10
	A. baumannii	1	2.0-9.2 × 10^7	0/1	0/1	0/1	0/1
	S. aureus MRSA	5	2.1-7.0 × 10^7	0/5	0/5	0/5	0/5
	S. aureus MSSA	6	1.7-5.3 × 10^7	0/6	0/6	0/6	0/6
	E. faecalis	8	3.8-6.9 × 10^7	0/8	0/8	0/8	0/8
	E. feacium	2	2.0-16 × 10^7	0/2	0/2	0/2	0/2
	E. avium	1	1.1-11 × 10^7	0/1	0/1	0/1	0/1
Fungi	*C. albicans*	10	1.4-16 × 10^7	0/10	0/10	0/10	5/10
	C. grabrata	2	4.7-7.2 × 10^7	0/2	0/2	0/2	2/2
	C. kurusei	1	3.7-7.1 × 10^7	0/1	0/1	0/1	1/1
	C. tropicalis	1	1.1-16 × 10^7	0/1	0/1	0/1	1/1

The available chlorine concentration was 50ppm
Contact time was 15 s. Detection limit was 0.5 CFU/ml

hydrophobic lipid bilayer. Thus, ClO^- exerts an oxidizing action only from outside of the cell membrane. On the other hand, molecular HClO in the weak acid region can penetrate the lipid bilayer of the plasma membrane by passive diffusion due to its electrical neutrality and its modest molecular size.

Figure 1 shows the relation between the ratio of HClO in the solution and the time for the first negative results against *B. cereus*, *B. subtilis* and *A. niger* under 50ppm chlorine concentrations. According to Figure 1, there is strong correlation between the ratio of HClO, and the relational coefficients (r^2) were 0.95, 0.98, and 0.99 for *B. subtillis*, *B. cereus*, and *A. niger,* respectively. This result indicates that microbicidal effect of hypochlorous solutions do not depend on the available chlorine concentration but on the HClO concentration, and it agrees with the results of previous research (Fukuzaki et al., 2007). A steep slope of the approximation formula of the figure means that the microorganism has high pH sensitivity under the treatment with a hypochlorous solution. That slopes for *B. cereus* and *B. subtilis* are steeper than that for *A. niger*, means that pH adjustment to weak acid region could kill spore-forming bacteria more effectively. It seems that the difference in the pH sensitivity is due to the hydrophobicity, the surface electric potential, and the microscopic structure of its membrane surface (Busscher et al., 1984; Isobe, 2001; Wiencek

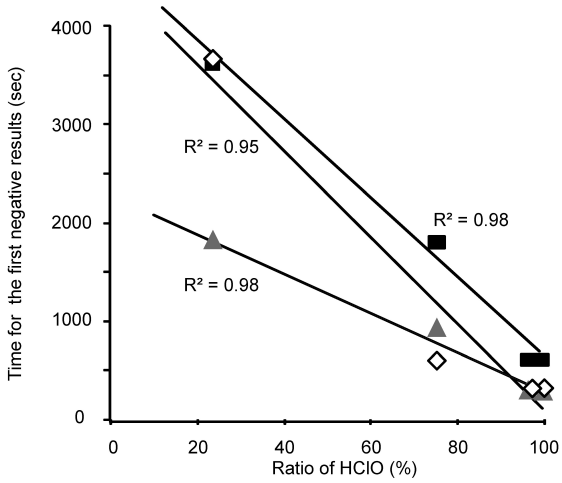

FIG. 1. A relation between the ratio of HClO in the solution and microbicidal effects.
*available chlorine concentration was 50ppm.
Symbols ; ■, *B. cereus* ; ◇, *B. subtilis* ; ▲, *A. niger*.

et al., 1990). In addition, among the test strains which have a low resistance to hypochlorous solutions, the difference in microbicidal effects due to pH values was not found. However, it may be affected by the pH range under lower available chlorine concentration conditions.

Most of the strains, that were used in this study were those involved in nosocomial infections (Ohara et al., 2007), and some of them have acquired drug resistance (Maesaki, 2010; NNIS system, 2004; Yoshikawa and Sasakawa, 2001). Instead of the overuse of antibiotics, environmental improvement is required for proper disinfection to control nosocomial infections in hospitals. In that case, the weak acid hypochlorous solution proposed by us has practical applicability because it has microbicidal effects at low concentrations and in a short contact time.

REFERENCES

Busscher, H., Weerkamp, A., Van der Mei, H., Antoon, W.J., Hans, P., and Joop, A. (1984) Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. *Appl. Environ. Microbiol*., **48**, 980-983.

Fukuzaki, S. (2006) Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. *Biocontrol Sci*., **11**, 147-157.

Fukuzaki, S., Urano, H., and Yamada, S. (2007) Effects of pH on the efficacy of sodium hypochlorite solution as cleaning and bactericidal agents. *Surface Sci*., **58**, 465-469.

Hayashi, Y. (2010) Current Topics of *Norovirus* Foodborne Disease (in Japanese). *Jpn. J. Food Microbiol*., **27**, 109-114.

Isobe, K. (2001) The Strategy for Existence of Microorganisms - Adhesion to Solid Surfaces—. *Surface Sci*., **22**, 652-662.

Leahy, J. G., Rubin, A. J., and Sproul, O. J. (1987) Inactivation of *Giardia muris* Cysts by free chlorine. *Appl. Environ.Microbiol*., **53**, 1448-1453.

Le Dantec, C., Duguet, J., Montiel, A., Dumoutier, N., Dubrou, S., and Vincent, V. (2002) Chlorine disinfection of atypical mycobacteria isolated from a water distribution system. *Appl. Environ. Microbiol*., **68**, 1025-1032.

Leyer, J. G., and Johnson, E. A. (1997) Acid adaptation sensitizes *Salmonella typhimurium* to hypochlorous acid. *Appl.Environ.Microbiol*., **63**, 461-467.

Maesaki, S. (2010) The clinical problem of the drug resistant bacterial infection in emergency units (in Japanese). *Nihon Kyukyu Igakugkai Zasshi*, **21**, 51-62.

Nakagawa, S., Goto, T., Nara, M., Ozawa, Y., Hotta, K., and Arata,Y. (1998) Spectroscopic characterization and the pH dependence of bactericidal activity of the aqueous chlorine solution. *Analytical Sci*., **14**, 691-698.

NNIS System, (2004) National nosocomial infections surveillance (NNIS) system report, data summary from January 1992 through June 2004, issued October 2004. *Amer. J. Infect*., **32**, 470-485.

Ohara, H., Nguyen, V. H., Truong, A., T., and Tran, Q. (2007) Report on Japan-Vietnam collaboration in nosocomial infection control at Bach Mai Hospital, Hanoi from 2000 to 2006. *Trop. Med. Health*, **35**, 253-259.

Ono, T. (2009) Measurement, assessment, and control of bacteria in manufacturing environment (10): Case study on the sterilization of environmental fungus with chlorinated disinfectants (in Japanese). *Bokin Bobai*, **37**, 129-138.

Ono, T., Miyake, M., and Yamashita, K. (2006a) Disinfection effect on vegetables by weak acid hypochlorous water to which a nonionic surfactant was added (in Japanese). *Bokin Bobai*, **33**, 257-262.

Ono, T., Miyake, M., and Yamashita, K. (2006b) Studies on the disinfection of hatching eggs by spraying weak acid hypochlorous water mist (in Japanese). *Bokin Bobai*, **34**, 465-469.

Ono, T., Yamashita, K., and Sato, T. (2010) Bactericidal effects of weak acid hypochlorous solution against various spores (in Japanese). *Bokin Bobai*, **38**, 509-514.

Sunayama, S., Sato, T., Kawamoto, A., Ohkubo, A., and Suzuki, T. (2002) Disinfection effect of hydrotalcite compounds containing antimicrobial metals agains microorganisms in water. *Biocontrol Sci*., **7**, 75-81.

Shinagawa, K. (2010) Foodborne disease outbreaks reported in Japan,1952-2009 —Outbreaks of microbial foodborne disease— (in Japanese). *Syokuhin Eiseigaku Zasshi*, **51**, 274-278.

Tomono, K. (2010) Infection control and prevention in hospitals (in Japanese). *Sogo Shinryo*, **59**, 431-434.

Urano, H., and Fukuzaki, S. (2005) The mode of action of sodium hypochlorite in the cleaning process. *Biocontrol Sci*., **10**, 21-29.

Wang, L., Bassiri, M., Najafi, R., Najafi, K.., Yang, J., Khosrovi, B., Hwong, W., Barati, E., Belisle, B., Celeri, C., and Robson, M. (2007) Hypochlorous acid as a potential wound care agent part I. stabilized hypochlorous acid: A component of the inorganic armamentarium of innate immunity. *Burns and Wounds*, **11**, 65-79.

Weber, D., Rutala, W., Miller, M. A., Huslage, K., and Sickbert-Bennett, E. (2010) Role of hospital surfaces in the transmission of emerging health care associated

pathogens: *Norovirus*, *Clostridium difficile*, and *Acinetobacter* species. *Amer. J. Infection Control*, **38**, S25-S33.
WHO (2008) Guidelines for drinking-water quality, third edition. pp.325.
Yoshikawa, M., and Sasakawa, C. (2001) *Ika Saikingaku*, (in Japansese), pp.99-133, Nankodo, Tokyo.
Wiencek, K. M., Klapes, N. A., and Foegeding, P. M. (1990) Hydrophobicity of *Bacillus* and *Clostridium* spores. *Appl. Environ. Microbiol*., **56**, 2600-2605.