Biocontrol Science, 2017, Vol. 22, No. 4, 223—227

Note

Stability of Weakly Acidic Hypochlorous Acid Solution with Microbicidal Activity

MASAYUKI ISHIHARA[1]*, KAORU MURAKAMI[2], KOICHI FUKUDA[1], SHINGO NAKAMURA[1], MASAHIRO KUWABARA[3], HIDEMI HATTORI[4], MASANORI FUJITA[5], TOMOHARU KIYOSAWA[3], AND HIDETAKA YOKOE[2]

[1]*Division of Biomedical Engineering, Research Institute, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-1324, Japan*
[2]*Department of Oral and Maxillofacial Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan*
[3]*Department of Plastic and Reconstructive Surgery, National Defense Medical College, Tokorozawa, Saitama 359-1324, Japan*
[4]*Department of Biochemistry and Applied Biosciences, University of Miyazaki, Miyazaki 889-2192, Japan*
[5]*Division of Environmental Medicine, Research Institute, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-1324, Japan*

Received 20 August, 2016/Accepted 13 February, 2017

Hypochlorous acid (HOCl) solution (200 ppm, pH 6) was prepared and evaluated for their stabilities and microbicidal activities. We demonstrated that HOCl is unstable against ultraviolet (UV) light, sunshine, contact with air, and elevated temperature (≧25℃). Furthermore, in the HOCl solution, the presence of excess NH_2- or CHO-containing organic compounds such as proteins and carbohydrates, or of inorganic ions such as NO_2^-, SO_3^-, PO_3^-, Fe^{2+}, Cu^{2+}, and CuS, resulted in the rapid consumption of HOCl by oxidation reactions, and significantly decreased the microbicidal activity of the HOCl solution against coliform bacteria and total viable cell count. Thus, production of stable HOCl solution requires formulation in pure water harboring concentrations as low as possible of various compounds and ions, as well as storage in dark and cool conditions (<10℃) to maintain the concentration of HOCl molecules and microbicidal activity.

Key words：Hypochlorous acid / Sodium hypochlorite / Microbicidal activity / Disinfectant / Stability.

The recent outbreak of Ebola virus hemorrhagic fever was a subject of concern worldwide. Once infected by this virus, many patients lost their lives, whether young or old (Xi et al., 2016). Indeed, there are many emerging pathogenic bacteria and viruses (such as norovirus, influenza virus, coronavirus, etc.) surrounding us (Song et al., 2015, Hakim et al., 2015, Ishihara et al., 2015). In the past, millions have lost their lives due to the presence of pathogenic microorganisms in the daily environment. Of especial concern is the need to protect vulnerable children in local communities of African and south and east Asian countries where infectious diseases are endemic. Many microbicidal compounds, including chlorine-based sanitizer (Ingram et al., 2003, Inagaki et al., 2011) have been the subject of study and development.

Sodium hypochlorite (ClO^-) solution (pH≧8) is a microbicide that shows strong oxidizing properties. Given this reagent's antimicrobial activity and low cost, ClO^- is widely used for sanitation and decontamination in various fields, including healthcare and food processing (Fukuzaki et al., 2006, Ardizzoni et al., 2009, Horiuchi et al., 2015). However, ClO^- has relatively poor antimicrobial activities against some microorganisms such as *Aspergillus oryzae*, bacterial spores, poliovirus, and norovirus. High concentrations (>1000 ppm) of ClO^- are generally recommended in clinical settings for the

*Corresponding author. Tel: +81-429-95-1601, Fax: +81-429-91-1611, E-mail : ishihara(a)ndmc.ac.jp

inactivation of microorganisms in spilled body fluids such as blood and feces, although at these concentrations the compound may produce carcinogens and poisonous substances resulting from its reaction with organic molecules. On the other hand, Hydrochlorous acid (HOCl) solution (pH 6) is superior to ClO^- solution in terms of higher microbicidal activity against a broad range of microorganisms (Horiuchi et al., 2015). However, HOCl is less stable to various environmental factors than is ClO^-. Notably, the presence of various organic compounds and inorganic ions results in rapid consumption of HOCl by oxidation reactions. HOCl has not been developed as a commercial pharmaceutical formulation, presumably because of the challenge of maintaining stability during storage, although there are few published study on the storage stability of HOCl solutions. Hence, the aim of this study was to identify methods for preparation and storage stabilization of HOCl solution; such methods are expected to permit the use of this reagent in clinical situations, such as the prevention and treatment of infection via environmental hygiene (Park et al., 2007, Inagaki et al., 2011) and in healing-impaired wounds (Sakarya et al., 2014).

HOCl (200 ppm, pH 6) and ClO^- (200 ppm, pH 8) solutions were prepared by the dilution of 0.5% ClO^- (Yoshida Pharmaceutical Corp., Tokyo, Japan) at 1/25 (v/v) into various test waters adjusted to pH to 5.5 and 7.5, respectively. The pH of 200 ppm HOCl or ClO^- solutions was adjusted to pH 6 or 8 with 1 N HCl or 1 N NaOH, respectively. The concentrations of HOCl and ClO^- were measured as residual chlorine levels using ClO (HOCl and ClO^-) -selective test papers (high concentration, 25 – 500 ppm; low concentration, 1 – 25 ppm; Kyoritu Check Laboratory Corp., Tokyo, Japan).

In an air contact study, HOCl solutions were formulated in pure water, tap water, or commercially available natural drinking water and then distributed into tissue culture dishes (100×20 mm; BD Falcon, Franklin Lakes, NJ, USA). To enhance air contact, the open dishes were covered with thin aluminum foil rather than the standard lids and allowed to incubate for up to 3 weeks on the lab bench-top at room temperature. For air shielded controls (i.e., with decreased air contact), dishes carrying the solutions were lidded and wrapped with a parafilm (Nikkei Products Co., Ltd., Tokyo, Japan). For all three water types, residual chlorine levels in HOCl solutions in the open dishes decreased more rapidly than those in the air shielded controls (FIG.1A). Notably, the decreases of residual chlorine levels in HOCl solutions formulated in pure water were attenuated compared to those in samples formulated in tap water and in commercial natural water; this pattern was observed in both open and air shielded dishes.

The effects of UV and sunshine irradiations on decrease of residual chlorine levels were also evaluated (FIG.1B). The HOCl solution was dispensed (at 10 mL/dish) into tissue culture dishes, which then were covered with thin plastic food wrap (Kureha Corp., Tokyo, Japan) in place of the lid. The UV irradiation was performed using a UV lamp (GL-15; Hitachi Appliance Inc., Tokyo, Japan) at a distance of 50 cm on a laminar flow cabinet (Air Tech Japan Ltd., Tokyo, Japan). The direct sunshine irradiation was performed by leaving it outside (32℃) on a fine weather day in summer. For

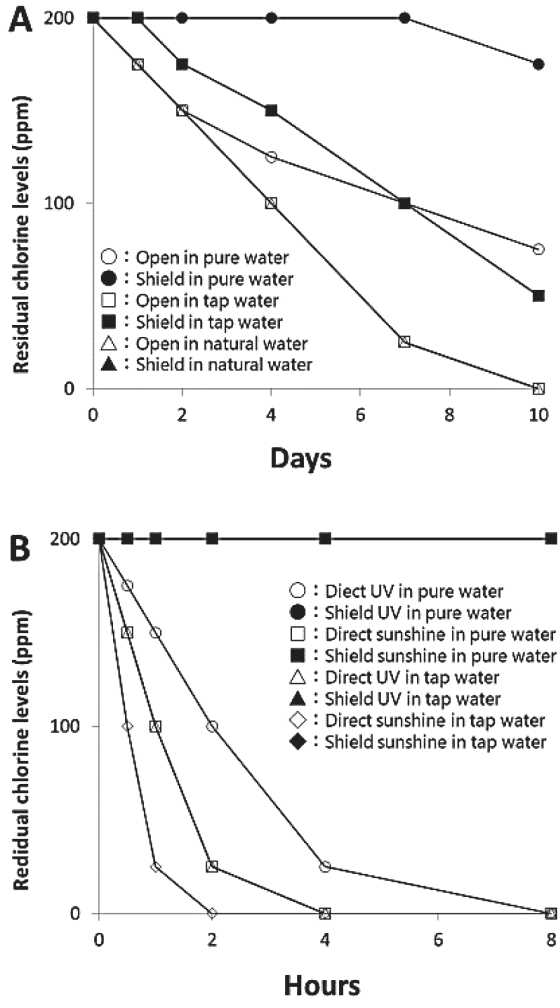

FIG. 1. Effects of increased air contact (A) and UV and sunshine irradiation (B) on residual chlorine levels. HOCl solutions (200 ppm, pH 6) were distributed in tissue culture dishes that were covered with aluminum foil ("open"; to permit air exchange) and lidded and sealed with Parafilm ("shielded" control) before being placed on the bench-top (A). HOCl solutions (200 ppm, pH 6) in the tissue culture dishes were covered with thin plastic food wrap for direct exposure, or with aluminum foil for shielded exposure (B). Residual chlorine levels were determined semi-quantitatively using high- and low-concentration ClO (HOCl and ClO^-) -selective test papers. Values are presented as means (n = 3).

the controls, the solution was shielded from illumination by covering the dishes with lids and aluminum foil before placing the dishes on the same clean bench. The decrease of residual chlorine levels in HOCl solutions formulated in pure water by direct irradiation of UV light and sunshine was attenuated compared to the decrease in those formulated with tap water. There were no decreases in residual chlorine levels in the HOCl solutions during 8 h of shielded exposure to both UV and sunshine irradiations, whether formulated with pure water or with tap water.

The half-life periods of residual chlorine levels (200 → 100 ppm) in HOCl solutions formulated with pure water, tap water, and filter paper-filtered pond water at 4℃ were 24 weeks, 8 weeks, and 2 weeks, respectively, while they in ClO^- solutions were >24 weeks, 20 weeks, and 4 weeks, respectively. The use of pure and cold water without contaminating compounds and ions significantly increased the half-life periods of residual chlorine levels in both HOCl and ClO^- solutions. Furthermore, the presence of excess organic compounds such as proteins and carbohydrates significantly reduced residual chlorine levels in both HOCl and ClO^- solutions. When Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Oriental, Tokyo, Japan) without fetal bovine serum and antibiotics was added into solutions of either HOCl or ClO^- formulated at 200 ppm with pure water and incubated at 37℃, residual chlorine levels in the solutions rapidly decreased, with the rate apparently depending on the concentration of DMEM. The decreases in HOCl molecules were faster than those seen for ClO^-. Notably, HOCl molecules were not detectable after a 1- day incubation of the HOCl solution in the presence of 4% (vol/vol) or more DMEM.

DMEM is composed of various minerals, carbohydrates, amino acids, and vitamins. We sought to determine which of these components are responsible for the depletion of HOCl and ClO^- molecules in solution. The presence of excess NH_2-containing organic compounds such as albumin, L-glycine, chitosan, glucosamine, and protamine rapidly decreased the residual chlorine levels of both HOCl and ClO^- (TABLE 1). The presence of excess CHO-containing organic compounds such as formalin, or of oxidizable inorganic compounds and ions such as NO_2^-, SO_3^-, PO_3^-, Fe^{2+}, Cu^{2+}, and CuS, also appeared to result in rapid consumption of HOCl and ClO^- by oxidation reaction. Thus, retention of HOCl levels appears to require formulation with pure water containing organic and inorganic compounds and ions at levels as low as possible.

Microbicidal activities of various concentrations of HOCl and inactivated HOCl were determined using filter paper-filtered pond water (pH 6) from a garden on the campus of National Defense Medical College. The inactivated HOCl solutions were prepared by diluting DMEM at 1/20 (vol/vol) into the HOCl solution and incubating the mixture for 24 h at 37℃; residual chlorine levels of the resulting solutions were below the limit of detection (< 1 ppm). The filtered pond water was diluted 1/2 and 1/20 into solutions of HOCl (at the indicated concentrations) or of inactivated HOCl, and the mixtures were incubated for 30 min at 25℃ on the bench-top. Aliquots (1 mL of each mixture) then were gently poured into individual dishes containing pre-aliquoted portions of simple and easy dry medium for coliform or for total viable count (Nissui Pharmaceutical Co., Ltd.). The resulting plates were incubated for 24 h in a 37℃ incubator (Alp Co., Ltd.), at which time the number of colonies in each dish was counted. Plating and counting was performed as a set of 4 technical replicates (n = 4).

The filter paper-filtered pond water (pH 6) contained 3220 coliform/mL and 2360 total viable count/mL, and the alkalified water (pH 8) contained only 11 coliform/mL and 72 total viable count/mL. Thus, alkalified pond water resulted in significant decreases of coliform bacteria and viable organisms. The addition of HOCl (>

TABLE 1. Half-life periods of residual chlorine levels in the presence of various compounds.

	pH 6	pH 8
L-glycine	1 h	2 h
Albumin	1 h	2 h
Chitosan	2 h	4 h
Glucosamine	4 h	8 h
$NaNO_2$	< 10 min	< 10 min
$NaNO_3$	2 days	7 days
KNO_2	<< 10 min	<< 10 min
KNO_3	2 days	7 days
NH_4Cl	10 min	2 h
Na_2SO_3	<< 10 min	<< 10 min
$Na_2S_2O_4$	<< 10 min	<< 10 min
Na_2PO_3	<< 10 min	<< 10 min
L-ascorbic acid	<< 10 min	<< 10 min
Iron (II) chloride	<< 10 min	30 min
CuS	30 min	30 min
Protamine	<< 10 min	<< 10 min
Formalin	1 day	1 day

Half-life periods of residual chlorine levels in ClO^- (pH 6) and HOCl (pH 8) solutions formulated with pure water and supplemented with excess concentrations of various organic and inorganic compounds (5 mg/mL, Formalin: 0.25%) were estimated at 37℃.

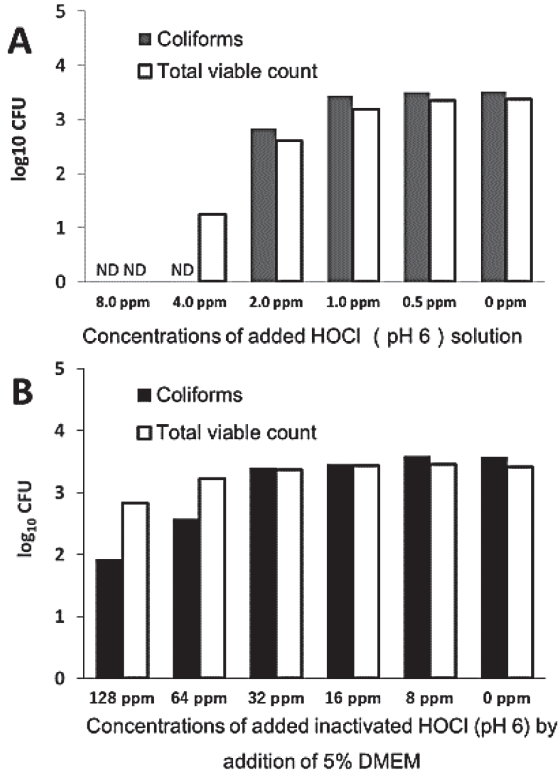

FIG. 2. Microbicidal activities of HOCl (A) and inactivated HOCl (B).
Microbicidal activities of various concentrations of HOCl (A) and inactivated HOCl (B) were determined using filter paper-filtered pond water Mixtures of pond water with HOCl or with inactivated HOCl were incubated at 37℃ for 24 h and plated for enumeration of colonies. Values are presented as means (n = 4).

4 ppm) to the pond water provided complete microbicidal activity against both coliform and total viable counts, while the addition of HOCl (≦4 ppm) showed partial antimicrobial activity in a concentration-dependent manner (FIG.2A). In fact, after incubating 2 and 1 ppm NaClO in the pond water (pH 6) for 30 min at 25℃, the levels of residual chloride were 1.0 ppm and ND (non-detected), respectively. Addition of 20 ppm ClO^- to the alkalified pond water (pH 8) yielded 7 coliform/mL and 55 total viable count/mL. On the other hand, inactivated HOCl (generated by incubating HOCl with 5% DMEM for 24 h at 37℃) harbored no detectable residual chlorine and exhibited very weak antimicrobial activity in concentrations of more than 32 ppm. Literature suggests that HOCl could be interacting with primary amino-groups ($-NH_2$) in organic compounds such as amino acids, thereby generating chloramine groups ($-NH_2Cl$ or $-NHCl_2$) that are known to show oxidizing properties and exhibit antimicrobial activity (Thomas et al., 1986, Korich et al., 1990, Gottardi et al., 2013). Consistent with the role of the chlorine moieties, the inactivated HOCl showed little antimicrobial activities even with inactivated HOCl (≧32 ppm) (FIG.2B).

Sodium hypochlorite (ClO^-) solution is widely used as a germicidal disinfectant in the fields of medicine and food production. ClO^- solution is dissociated in alkaline water (pH≧8) into hypochlorous ion (ClO^-), sodium hydroxide (NaOH), and small amounts of hydrochlorous acid (HOCl). Of those substances, HOCl is well known to possess microbicidal properties, and the HOCl molecule becomes the predominant component in weakly acidic solution (pH: 4-6.5). The present study showed that solutions of HOCl are less stable to UV irradiation, sunshine, contact with air, and elevated temperature (>25℃) than ClO^- solutions. The storage stability of HOCl solution requires low temperatures (<10℃), shielding from light, and minimal air contact. Furthermore, the presence of various NH_2- or CHO-containing organic compounds such as proteins, amino acids, and carbohydrates, or inorganic compounds and ions such as NO_2^-, SO_3^{2-}, PO_3^{2-}, Fe^{2+}, Cu^{2+}, and CuS, rapidly reduced the microbicidal activity of HOCl solutions, as the result of rapid consumption of HOCl by oxidation reactions. Thus, stable HOCl production requires pure water containing concentrations as low as possible of organic and inorganic compounds and various ions. HOCl have not gained approval from the Pharmaceuticals and Medical Devices Agency for use as a pharmaceutical or medical devices, despite the approval of NaClO for this purpose, presumably because of the challenge of maintaining storage stability; there have been (to our knowledge) few studies on the storage stability of HOCl solutions.

The microbicidal effect of HOCl solution has been well studied (Kim et al., 2008, Hao et al., 2013). This reagent has broad-spectrum microbicidal activity. HOCl engages in an irreversible reaction with sulfur- and heme-containing membrane enzymes and structural proteins; the resulting damage causes loss of respiratory function in the bacterial cell membrane, leading in turn to nonviability and cell death (Ono et al., 2012, Fukuzaki et al., 2006). For example, 50 ppm HOCl provides a > 3-log decrease in the counts of pathogenic *E coli*, *E coli* O-157, *Staphylococcus aureus*, MRSA (Methicillin-resistant *Staphylococcus aureus*), Salmonellae, and *Pseudomonas aeruginosa* (Tachikawa et al., 1999, Ono et al., 2012); compounds such as alkaline ClO^- and benzalkonium chloride require concentrations of 200 ppm and 500 ppm, respectively, to achieve similar antibacterial activities (Nemoto et al., 2014). Furthermore, ClO^- and benzalkonium chloride have little bactericidal activity against the bacterial spores of *Bacillus subtilis*. Thus, HOCl is superior to ClO^- in terms of microbicidal

activity, and HOCl also offers the possibility of use as a spray in a room space. In the present study, the addition of > 4 ppm HOCl to pond water (pH 6) containing thousands of bacterial CFUs provided complete microbicidal activity against coliforms and total viable counts, while the addition of ≦4 ppm HOCl showed only partial antimicrobial activity. Thus, the microbicidal activities of HOCl solutions correlate inversely with the levels of the residual chlorine.

In conclusion, HOCl solution (pH 6) should be stored in cool (<10℃), dark places and contact with air should be minimized. Furthermore, the production of active HOCl solutions requires formulation in pure water that contains concentrations as low as possible of organic and inorganic compounds and various ions; formulated solutions should be used within a few weeks in tropical regions, including Africa and south and east Asia. It may be desirable, for on-site-production of HOCl solutions, to employ water-purifying systems that can remove organic and inorganic compounds and ions from the contaminated water. The resulting HOCl are expected to enable improved environmental hygiene and to serve as a potential wound healing agent.

REFERENCES

Ardizzoni, A., Blasi, E., Rimoldi, C., Giardino, L., Ambu, E., Righi, E., and Neglia, R. (2009) An *in vitro* and *ex vivo* study on two antibiotic-based endodontic irrigants: a challenge to sodium hypochlorite. *New Microbiologica*, **32**, 57-66.

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

Gottardi, W., Debabov, D., and Nagl, M. (2013) N-chloramine, a promissing class of well-tolerated topical anti-infectives. *Antimicrob. Agents Chemother*, **57**, 1107-1114.

Hakim, H., Thammakarn, C., Suguro, A., Ishida, Y., Kawamura, A., Tamura, M., Satoh, K., Tsujimura, M., Hasegawa, T., and Takehara, K. (2015) Evaluation of sprayed hypochlorous acid solutions for their virucidal activity against avian influenza virus through *in vitro* experiments. *J. Vet. Med. Sci*., **77**, 211-215.

Hao, X. X., Li, B. M., Zhang, Q., Lin, B. Z., Ge, L. P., Wang, C. Y., and Cao, W. (2013) Disinfection effectiveness of slightly acidic electrolyzed water in swine barns. *J. Appl. Microbiol*., **115**, 703-710.

Horiuchi, I., Kawata, H., Nagao, T., Imaohji, H., Murakami, K., Kino, Y., Yamasaki, H., Koyama, A. H., Fujita, Y., Gota, H., and Kuwahara, T. (2015) Antimicrobial activity and stability of weakly acidified chlorous acid water. *Biocontrol Sci*., **20**, 43-51.

Ingram, P. R., Homer, N. Z. M., Smith, R. A., Pitt, A. R., Wilson, C. G., Olejnik, O., and Spickett, C. M. The interaction of sodium chlorite with phospholipids and glutathione: a comparison of effects *in vitro*, in mammalian and in microbial cells. *Arch. Biochem. Biphys*., **410**, 121-133.

Ishihara, M., Nguyen, V. Q., Mori, Y., Nakamura, S., and Hattori, H. (2015) Adsorption of silver nanoparticles onto different surface structures of chitin/chitosan and correlations with antimicrobial activities. *Int. J. Mol. Sci*., **16**, 13973-13988.

Inagaki, H., Shibata, Y., Obata, T., Kawagoe, M., Ikeda, K., Sato, M., Toida, K., Kushima, H., and Matsuda, Y. (2011) Effects of slightly acidic electrolyzed drinking water on mice. *Lab. Anim*., **45**, 283-285.

Kim, H. J., Lee, J. G., Kang, J. W., Cho, H. J., Kim, H. S., Byeon, H. K., and Yoon, J. H. (2008) Effects of a low concentration hypochlorous acid nasal irrigation solution on bacteria, fungi, and virus. *Laryngoscope*, **118**, 1862-1867.

Kobayashi, M., Akiyama, S., Iwashita, M., and Suzuki, A. (1989) A study of the bactericidal effect of a sodium chlorite preparation. *J. Food Hyg. Soc. Jap*., **30**, 367-374.

Korich, D. G., Mead, J. R., Madore, M. S., Sinclair, N. A., and Sterling, C. R. (1990) Effects of ozone, chlorine dioxide, chlorine, and monochloramine on cryptosporidium parvum oocyst viability. *Appl. Environ. Microbiol*., **56**, 1423-1428.

Nemoto, M., Bannai, H., Tsujimura, K., Yamanaka, T., and Kondo, T. (2014) Virucidal effect of commercially available disinfectants on Equine group A Rotavirus *J. Vet. Med. Sci*., **76**, 1061-1063.

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.

Park, G. W., Boston, D. M., Kase, J. A., Sampson, M. N., and Sobsey, M. D. (2007) Evaluation of liquid- and fog-based application of Sterilox hypochlorous acid solution for surface inactivation of human norovirus. *Appl. Environ. Microbiol*., **73**, 4463-4468.

Sakarya, S., Gunay, N., Karakulak, M., Ozturk, B., and Ertugrul, B. (2014) Hypochlorous acids: an ideal wound care agent with powerful microbicidal, antibiofilm, and wound healing potency. *Wounds*, **26**, 342-350.

Song, J. Y., Cheong, H. J., Choi, M. J., Jeon, J. H., Kang, S. H., Jeong, E. J., Yoon, J. G., Lee, S. N., Kim, S. R., Noh, J. Y., and Kim, W. J. (2015) Viral Shedding and Environmental Cleaning in Middle East Respiratory Syndrome Coronavirus Infection. *Infect. Chemother*., **47**, 252-255.

Tachikawa, T., Watari, E., Someya, K., Ikeda, T., Araaki, M., Fujimaki, W., Kanai, T., Uchiyama, T., and Miyanaga, Y. (1999) Efficacy of a weak acid electrolytic water against various kinds of pathogenic microorganisms. *Japanese Journal of Infection and Control*, **14**, 255-263.

Thomas, E. L., Grisham, M. B., Jefferson, M. M. (1986) Preparation and characterization of chloramines. *Methods Enzymol*., **132**, 569-585.

Xi, H., Cao, J., Liu, J., Li, Z., Kong, X., Wang, Y., Chen, J., Ma, S., and Zhang, L. (2016) Improving health care workers' protection against infection of Ebola hemorrhagic fever through video surveillance. *Am. J. Infect. Control*., **44**, 922-924.