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Bactericidal efficacies of food additive grade calcium hydroxide toward *Legionella pneumophila*

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ABSTRACT. Food additive grade calcium hydroxide ($FdCa(OH)_2$) in the solution of 0.17% was evaluated for its bactericidal efficacies toward *Legionella pneumophila* with or without sodium hypochlorite (NaOCl) at a concentration of 200 ppm total residual chlorine, at room temperature (RT) (25°C ± 2°C) and 42°C, either with or without 5% fetal bovine serum (FBS). Besides, $FdCa(OH)_2$ in different concentration solutions were prepared in field water samples (hot spring and bath tab water) and evaluated for their bactericidal efficacies at 42°C. $FdCa(OH)_2$ (0.17%) inactivated the *L. pneumophila* to the undetectable level (<2.6 log CFU/m*l*) within 5 min and 3 min, respectively, at RT and 42°C, with 5% FBS. At RT and 42°C, NaOCl inactivated *L. pneumophila* to the undetectable level within 5 min, without 5% FBS, but with 5% FBS, it could only inactivate this bacterium effectively (≥3 log reductions). Conversely, at RT and 42°C, the mixture of 0.17% $FdCa(OH)_2$ and 200 ppm NaOCl could inactivate *L. pneumophila* to the undetectable level, respectively, within 3 min and 1 min, even with 5% FBS, and it was elucidated that $FdCa(OH)_2$ has a synergistic bactericidal effect together with NaOCl. $FdCa(OH)_2$ 0.05% solution prepared in hot spring water could inactivate *L. pneumophila* to the undetectable within 3 min at 42°C. So, $FdCa(OH)_2$ alone could show nice bactericidal efficacy at 42°C, even with 5% FBS, as well as in field water samples.

KEY WORDS: bactericidal activity, food additive grade calcium hydroxide ($FdCa(OH)_2$), *Legionella pneumophila*, sodium hypochlorite, synergistic effect

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The genus *Legionella* currently has at least 50 species comprising 70 distinct serogroups, many of which are considered pathogenic [15, 32, 33, 41]. In 1976, the most pathogenic species, *Legionella pneumophila* (*L. pneumophila*), was documented as the first recognized species of this genus following an outbreak of severe pneumonia among the participants of the American Legion convention, in Philadelphia [15, 16, 31]. In the U.S.A. and Europe, strains of *L. pneumophila* belonging to serogroup 1 are responsible for the most legionellosis cases in human [9, 15, 52]. Infection caused by *Legionella* bacteria is one of the major causes of community-acquired pneumonia [53]. Legionellosis is a disease acquired by the inhalation or aspiration of aerosolized water or soil (potting soil, compost soil) contaminated with *Legionella,* such as found in cooling towers, evaporative condensers of air-conditioning systems, whirlpool spas, showers, and hot water tanks [15, 33, 50]. Legionellosis may take shape of a pneumonia-type illness called Legionnaires' disease, or a mild flu-like illness called Pontiac fever.

In the U.S.A., during 2009 and 2010, Center for Disease Control and Prevention (CDC) reported that *Legionella* accounted for 19 of the 33 drinking water-related waterborne disease outbreaks, causing 72 illnesses and 8 deaths [11, 51]. In 2014, about 7,000 cases of detected Legionnaires' disease were reported in the European Union [14]. In Japan, more than 1,100 cases of legionellosis were reported in Infectious Agents Surveillance Report of 2014, caused by contaminated artificial whirlpool spas or natural hot springs [40]. In 2002, a major outbreak causing seven deaths out of 295 cases of infection originated from a newly opened hot

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spring spa, in Japan [39]. Thus, *Legionella* infection caused by contaminated bath water is a significant public health concern in Japan [26, 28, 36], and sensitive detection and identification of *Legionella* in bath water samples is crucial for the control of legionellosis.

Hot springs and public baths indeed constitute the major sources of legionellosis in Japan [27, 36, 37]. In 2015, an outbreak of Legionnaires' disease occurred among 7 people who had visited a spa house, due to *L. pneumophila* [27]. Compared to Europe and the U.S.A., there have been many cases of legionellosis in Japan in connection with hot spring spa and continuous circulating public and domestic hot water baths, caused not only by aerosolization, but also by aspiration of water [25]. *Legionella* can infect animals and develop disease, however animals have not been identified as carriers of *Legionella* [12].

Adequate and proper disinfection procedures can reduce the incidence of infectious diseases and their transmission. Several environmental factors such as organic materials, temperature, humidity and contact time with the pathogen are responsible for altering the kinetics of disinfection [22, 42]. A wide variety of disinfection methods are used for controlling the concentrations of *Legionella* sp. and to meet the water quality standards for each type of water system [24, 46]. In industry, chemical disinfectants, particularly oxidizing agents such as chlorine, chlorine dioxide, chloramine, and ozone are most widely used to control *Legionella* sp. and protozoa [23]. In drinking water systems, there are different approaches for the control of *Legionella*. Chemical disinfection, e.g. by chlorine compounds, does not result in the desirable antibacterial effect, due to the fact that *Legionellae* are often incorporated in biofilms and amoebae that physically protect the bacterium against direct contact with disinfectants [24].

Sodium hypochlorite (NaOCl) is a widely used popular disinfectant throughout the world, but the efficacy of NaOCl is inadequate due to the presence of organic materials [20, 49]. Food additive grade calcium hydroxide ($FdCa(OH)_2$) is an alkaline agent with high pH (in 0.17% solution, pH 12.7), relatively novel product among materials that can inactivate pathogens, and it did demonstrate strong bactericidal efficacies even in the presence of dust/feces, so as to prevent environmental contamination [18, 19, 43, 49]. According to the information of Fine Co., Ltd. (Tokyo, Japan), 0.2% $FdCa(OH)_2$ solution was evaluated by Japan Food Research Laboratories (Tokyo, Japan) to be in the "non-irritant" category, given the primary skin irritation test using rabbits according to OECD Guideline for the testing of Chemicals 420 (2002).

In the present study, we investigated the bactericidal efficacies of NaOCl and $FdCa(OH)_2$ alone and their combination at two different temperatures, namely room temperature (RT) (25 ± 2°C) and 42°C, with varying contact times either with or without organic loads in suspension tests toward *L. pneumophila*. In addition, the synergistic bactericidal efficacies between NaOCl and $FdCa(OH)_2$ were also compared with each single disinfectant. For the application of $FdCa(OH)_2$, hot spring water originated in a hotel was used as a diluent of $FdCa(OH)_2$, and evaluation of *Legionella* inactivation was carried out.

MATERIALS AND METHODS

Application of $FdCa(OH)_2$ powder

$FdCa(OH)_2$ powder is highly alkaline in nature, presented at pH 13 in 10% solution supernatant, and composed of natural calcium carbonates derived from limestone through calcination process, with average diameter of powder particles at 10 μm [19, 49]. It was kindly provided by Fine Co., Ltd. In the laboratory, 0.025, 0.05, 0.1 and 0.17% suspensions (w/v) of $FdCa(OH)_2$ were prepared by redistilled water (dW_2) and centrifuged at 1,750 × g for 10 min at RT. The resultant supernatants were used as tested solutions of 0.025, 0.05, 0.1 and 0.17% $FdCa(OH)_2$, respectively.

Application of NaOCl

NaOCl (Turukuron®, at 12% chlorine concentration) was purchased from Toagosei Co., Ltd. (Tokyo, Japan). NaOCl tested solution containing 200 ppm chlorine (200 ppm NaOCl) was freshly prepared in dW_2 and used in this study within 10 min after preparation.

Combination of NaOCl and $FdCa(OH)_2$

For the mixed solution (200 ppm NaOCl and 0.17% $FdCa(OH)_2$), NaOCl was diluted in 0.17% $FdCa(OH)_2$ solution (final concentration: 200 ppm chlorine in the solution).

During experiments at 42°C temperature, all solutions and bacteria were kept in hot water bath for at least 10 min prior to starting the experiment for maintaining reaction temperature 42°C (Fig. 1).

Hot spring and bath tab water samples

Freshly originated hot spring water from the ground (pH 7.8) and bath tab water (60% original hot spring water, plus 20% tap water, plus 20% recycled bath tab water; pH 7.2) were collected from a hotel located in Niigata prefecture. Due to the transparency of the bath bat water, the presence of organic materials seemed to be unremarkable. So the hot spring water and the bath tab water were used for the preparation of different concentration (0.05, 0.025 and 0.0125%) of $FdCa(OH)_2$ as described above, and thereby evaluate their bactericidal efficacies, while imitating field conditions.

Blocking solution

Chemical neutralizer, namely a blocking solution, was prepared by adding fetal bovine serum (FBS) in 1 M Tris–HCl (pH 7.2) (final 50% FBS and 0.5 M Tris-HCl), and used to stop bactericidal activity, after definite contact time of disinfectant application.

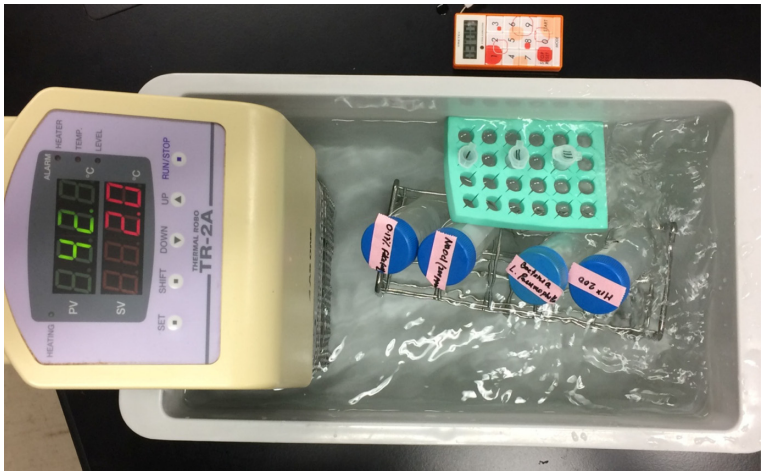

Fig. 1. Test solutions and bacteria were kept in hot water bath at temperature 42ºC for at least 10 min prior to start the experiment for maintaining treatment temperature and imitating field conditions.

Bacterial strain

L. pneumophilia 1 (No. 1675) on buffered charcoal-yeast extract agar plates with alpha-ketoglutarate (BCYEα), isolated from public bath water, was kindly supplied from Niigata Prefectural Institute of Public Health and Environmental Sciences (Niigata, Japan). It was originally detected in a hot spring bath tab water by filtration concentration method (acid treatment method) and determined by serotyping (Denka Seiken Co., Ltd., Tokyo, Japan). After receive the strain, it was confirmed by 16S ribosomal RNA sequence (accession number LC486880).

Preparation of bacterial suspension

L. pneumophilia was stocked in 10% skim milk at −80°C until used. Prior to the experiment, this bacterium was sub-cultured onto Wadowsky-Yee-Okuda agar plates containing 5 *μ*g/m*l* vancomycin and 100 U/m*l* polymyxin with alpha-ketoglutarate (WYO-alpha plates; Eiken Chemical Co., Ltd., Tokyo, Japan), and then incubated at 37°C for 72–96 hr. Following incubation, colonies were picked up and cultivated in Trypticase-Yeast Maltose (TYM) medium containing 2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.1% Glucose, 0.25% $MgSO_4$ and 0.5% NaCl (pH 7.0) and incubated at 37°C for 16 hr with 150 rpm shaking. Following incubation, TYM medium containing cultured bacteria was centrifuged (1,750 × g for 20 min) and re-suspended in PBS thrice for removing organic materials. The bacterial suspension was prepared with PBS and the number of bacteria (CFU/m*l*) was counted on WYO-alpha agar plates after making serial 10-fold dilution, followed by 72–96 hr incubation at 37°C.

Evaluating the bactericidal activities of the treatment solutions toward L. pneumophilla in aqueous phase

For estimating the inactivation capacity toward *L. pneumophilla* in aqueous phase, two different reaction temperatures (RT and 42°C) and a number of contact times were thereby investigated, either with or without presence of 5% FBS. At RT, in the absence of organic materials, four hundred micro-litters of each tested solution (0.17% $FdCa(OH)_2$, 200 ppm NaOCl and the mixture) was added to one hundred micro-litters of *L. pneumophilla* bacteria suspension in a micro-tube, vortexed and incubated at indicated times (0 sec, 30 sec, 1 min, 3 min and 5 min) at RT and 42°C. Following incubation, inactivation toward bacteria was stopped by adding 500 *μl* of the blocking solution. On the other hand, to assess the inactivating capacity of these solutions in the presence of organic materials, 25 *μl* of FBS (5% at the final concentration in the reaction) was added into the micro-tube containing 375 *μl* of the each tested solution, then 100 *μl* of bacteria was added and incubated for above mentioned contact time at RT or 42°C and at last inactivation was stopped as described above. The viable bacterial load in each sample then was counted (log_{10} CFU/m*l*). For the experiment at 42°C, the micro-tubes were incubated in a water bath to maintain this temperature. In addition, the efficacy of different concentrations (0.025, 0.05 and 0.1%) of $FdCa(OH)_2$ solutions (lower than 0.17%) were also evaluated at 42°C temperature, with the presence of 5% FBS using above mentioned way.

To confirm the effect of the blocking solution, the latter was added to each tested solution, before adding bacteria (considered 0 sec treatment). For the positive control, 100 *μl of L. pneumophila* was inoculated in 400 *μl* of PBS and 500 *μl* of the blocking solution. Then, the viable number of bacteria was counted (log_{10} CFU/m*l*).

Evaluating the bactericidal activities of the treatment solutions (prepared in original hot spring and hot spring bath tab water samples) toward L. pneumophilla at 42°C

For imitating field conditions, the inactivation efficacies toward *L. pneumophilla* were investigated, using the treatment solutions prepared in original hot spring and hot spring bath tab water at 42°C. Before the experiments, 200 m*l* of the water samples were centrifuged at 1,700 × g for 30 min, and resuspended in 500 *μl* dW_2. The concentrated samples were then tested for *Legionella*, but

no colony was observed on WYO-alpha agar plates.

One hundred micro-litters of freshly cultured *L. pneumophilla* bacteria suspension was added separately in a micro-tube containing 400 μl of (0.05, 0.025 and 0.0125%) $FdCa(OH)_2$ solutions (prepared in the hot spring water and the bath tab water), then mixed by vortex and incubated at 42°C for 3 min. Following incubation, inactivation was stopped by adding 500 μl of the blocking solution. The viable bacterial load in each sample was counted ($\log_{10}$ CFU/m*l*). For the experiment at 42°C, the micro-tubes were incubated in a water bath to maintain this temperature. Each solution was tested in triplicates, and the bacterial numbers were shown in mean ± standard error (SE).

Inactivation analysis

Inactivation efficacy toward the bacteria was determined by calculating the reduction factor (RF), using the following equation: RF=tpc −ta, where tpc is the bacteria number of the positive control or untreated sample in $\log_{10}$ units, and ta is the number of the recovered bacteria of the treated samples in $\log_{10}$ units. Inactivation was considered to be efficient when RF was ≥3, demonstrating a reduction of bacterial number greater than 1,000 times [29, 45, 48].

Statistical analysis

The RF values of each experiment were analyzed independently and shown as mean ± SE. For each condition, a one-way ANOVA (SPSS, IBM, Armonk, NY, U.S.A.) followed by Tukey's honest significant difference post hoc test was carried out to determine the statistical significance of differences in disinfection efficacy between the positive control and treatment group, and also among the treatment group. Significant difference was noted when the associated *P* value was less than 0.05.

RESULTS

Evaluation of bactericidal efficacies of tested solutions against L. pneumophilla in aqueous phase

Table 1 shows the bactericidal activities of 0.17% $FdCa(OH)_2$, 200 ppm NaOCl and their mixture in liquid against *L. pneumophilla,* at RT. When the blocking solution was added to the tested solution prior to adding the bacteria (0 sec), almost no bacterial reduction was revealed (*P*>0.05), comparing to positive control (Table 1), which ensured that the blocking solution stopped inactivation reaction by the tested solution absolutely.

At RT, as shown in Table 1, NaOCl could inactivate *L. pneumophila* effectively (RF≥3.0 $\log_{10}$ CFU/m*l* bacterial reduction) from 8.37 and 8.34 $\log_{10}$ CFU/m*l* to 3.36 (RF=5.01) and 3.86 (RF=4.48) $\log_{10}$ CFU/m*l* within 5 min and 1 min, respectively, with or without 5% FBS; but in the absence of 5% FBS, it required 5 min to inactivate this bacterium to the undetectable level (≤2.6 $\log_{10}$ CFU/m*l*) (RF≥5.74). However, Mix200 inactivated this bacterium to the undetectable level (≤2.6 $\log_{10}$ CFU/m*l*) within 3 min and 1 min, respectively, with or without 5% FBS; and effectively within 1 min (RF=4.05) and 30 sec (RF=3.79), respectively. $FdCa(OH)_2$ (0.17%) solution could inactivate the bacteria to undetectable level (≤2.6 $\log_{10}$ CFU/m*l*) within 5 min even with 5% FBS, but it required 3 min and 1 min for effective inactivation of bacteria, respectively, with or without FBS. Statistically significant (*P*<0.05) difference was noted between positive control and treatment group (when RF value of treatment group was ≥3 $\log_{10}$ CFU/m*l*).

At 42°C, Table 2 demonstrates the inactivation efficacy of NaOCl tested solution against *L. pneumophilla* was similar to RT within same contact time, either with or without 5% FBS. On the other hand, in the presence of 5% FBS, Mix200 and 0.17% $FdCa(OH)_2$ could inactivate this bacterium to the undetectable level (≤2.6 CFU/m*l*) (RF≥5.89), respectively, within 1 min and 3 min but for the effective inactivation, required 30 sec (RF=4.37) and 1 min (RF=4.51), respectively, as shown in Table 2. So, at high temperature, the bactericidal efficacy of Mix200 and 0.17% $FdCa(OH)_2$ tested solution was enhanced and required a very short contact time for the effective inactivation.

Table 3 shows the bactericidal efficacy of different concentrations of $FdCa(OH)_2$ (0.025, 0.05 and 0.1%) at 42°C with 5% FBS. It was found that 0.1% (RF=5.05) and 0.05% (RF=4.03) solution could inactivate *L. pneumophilla* bacteria effectively within 3 min, but 0.025% (RF=2.05) solution was not capable of inactivating the bacteria effectively (RF<3), even within 3 min.

Table 1. Bactericidal efficacies of 0.17% $FdCa(OH)_2$, 200 ppm NaOCl or the mixture solution toward *Legionella pneumophilla* at 25°C

Test solution	FBS[a] (%)	Bacterial titer [$\log_{10}$ (CFU/m*l*)] at different contact time					
		Positive control	0 sec	30 sec	1 min	3 min	5 min
$FdCa(OH)_2$[b]	0	8.34 ± 0.18[e]	8.14 ± 0.07	5.88 ± 0.37	4.86 ± 0.21*	2.90 ± 0.30*	≤2.60 ± 0.00
NaOCl[c]			8.20 ± 0.09	5.70 ± 0.55	3.86 ± 0.21*	2.76 ± 0.16*	≤2.60 ± 0.00
Mix200[d]			8.11 ± 0.08	4.55 ± 0.49*	≤2.60 ± 0.00	NT	NT
$FdCa(OH)_2$	5	8.37 ± 0.17	8.07 ± 0.10	NT[f]	5.40 ± 0.49	3.17 ± 0.30*	≤2.60 ± 0.00
NaOCl			8.09 ± 0.18	NT	6.75 ± 0.48	5.60 ± 0.66	3.36 ± 0.38*
Mix200			8.14 ± 0.07	5.80 ± 0.20	4.32 ± 0.38*	≤2.60 ± 0.00	NT

a) FBS: fetal bovine serum, b) $FdCa(OH)_2$: Food additive grade calcium hydroxide powder (170 mg) was prepared in 100 m*l* of redistilled water, c) NaOCl: Sodium hypochlorite solution containing 200 ppm chlorine (200 ppm NaOCl) was prepared in dW_2, d) Mix200: NaOCl was diluted to 200 ppm chlorine in 0.17% $FdCa(OH)_2$ solution, e) Bacterial count ($\log_{10}$ CFU/m*l*), f) NT: not tested. *Single asterisk indicates effective bacterial reduction (≥3 $\log_{10}$ CFU/m*l*). Bacterial titer ≤2.6 $\log_{10}$ CFU/m*l* indicates reduction to undetectable level. Both effective and bacterial reductions to undetectable level are significantly different (*P*<0.05) from positive control titer.

Table 2. Bactericidal efficacies of 0.17% $FdCa(OH)_2$, 200 ppm NaOCl or the mixture solution toward *Legionella pneumophilla* at 42°C

Tested solution	FBS[a] (%)	Bacterial titer [log_{10} (CFU/m*l*)] at different contact time					
		Positive control	0 sec	30 sec	1 min	3 min	5 min
$FdCa(OH)_2$[b]	0	8.52 ± 0.17[e]	8.38 ± 0.10	5.64 ± 0.11	3.68 ± 0.06*	≤2.60 ± 0.00	NT
NaOCl[c]			8.34 ± 0.05	6.30 ± 0.22	5.59 ± 0.31	3.50 ± 0.25*	≤2.60 ± 0.00
Mix200[d]			8.28 ± 0.11	3.56 ± 0.27*	≤2.60 ± 0.00	NT	NT
$FdCa(OH)_2$	5	8.49 ± 0.14	8.29 ± 0.11	5.64 ± 0.11	3.98 ± 0.51*	≤2.60 ± 0.00	NT
NaOCl			8.12 ± 0.08	NT[f]	6.39 ± 0.19	5.60 ± 0.20	3.18 ± 0.31*
Mix200			8.24 ± 0.12	4.12 ± 0.29*	≤2.60 ± 0.00	NT	NT

a) FBS: fetal bovine serum, b) $FdCa(OH)_2$: Food additive grade calcium hydroxide powder (170 mg) was prepared in 100 m*l* of redistilled water, c) NaOCl: Sodium hypochlorite solution containing 200 ppm chlorine (200 ppm NaOCl) was prepared in dW_2, d) Mix200: NaOCl was diluted to 200 ppm chlorine in 0.17% $FdCa(OH)_2$ solution, e) Bacterial count (log_{10} CFU/m*l*), f) NT: not tested. *Single asterisk indicates effective bacterial reduction (≥3 log_{10} CFU/m*l*). Bacterial titer ≤2.6 log_{10} CFU/m*l* indicates reduction to undetectable level. Both effective and bacterial reductions to undetectable level are significantly different (P<0.05) from positive control titer.

Table 3. Bactericidal efficacies of $FdCa(OH)_2$ solution with different concentrations toward *Legionella pneumophilla* at 42°C in the presence of 5% fetal bovine serum

Tested solution[a]	Bacterial titer [log_{10} (CFU/m*l*)]	
	Positive control	3 min contact time
0.1% $FdCa(OH)_2$	8.13 ± 0.04[b]	3.08 ± 0.48*
0.05% $FdCa(OH)_2$		4.10 ± 0.39*
0.025% $FdCa(OH)_2$		6.08 ± 0.28

a) Different concentrations of food additive grade calcium hydroxide [$FdCa(OH)_2$] tested solution was prepared in redistilled water, b) Bacterial count (log_{10} CFU/m*l*). *Single asterisk indicates effective bacterial reduction (≥3 log_{10} CFU/m*l*). Bacterial titer ≤2.6 log_{10} CFU/m*l* indicates reduction to undetectable level. Effective bacterial reductions are significantly different (P<0.05) from positive control titer.

Table 4. Bactericidal efficacies of different concentrations of $FdCa(OH)_2$ solution (prepared in field water samples) toward *Legionella pneumophilla* at 42°C

Tested solution[a]		Bacterial titer [log_{10} (CFU/m*l*)]	
		Positive control	3 min contact time
Prepared in hot spring water	0.05% $FdCa(OH)_2$	8.95 ± 0.29[b]	≤2.60 ± 0.00*
	0.025% $FdCa(OH)_2$		3.72 ± 1.12*
	0.0125% $FdCa(OH)_2$		5.61 ± 1.21
Prepared in bath tab water	0.05% $FdCa(OH)_2$		3.18 ± 0.58*
	0.025% $FdCa(OH)_2$		4.36 ± 1.06*
	0.0125% $FdCa(OH)_2$		6.58 ± 0.83

a) Different concentrations of food additive grade calcium hydroxide [$FdCa(OH)_2$] tested solution were prepared in hot spring water and bath tab water using same way described in the above tables, b) Bacterial count (log_{10} CFU/m*l*). *Single asterisk indicates effective bacterial reduction (≥3 log_{10} CFU/m*l*). Bacterial titer ≤2.6 log_{10} CFU/m*l* indicates reduction to undetectable level. Both effective and bacterial reductions to undetectable level are significantly different (P<0.05) from positive control titer.

Statistically significant (P<0.05) difference was found between positive control and treatment group (when RF≥3).

Table 4 demonstrated that $FdCa(OH)_2$ 0.025 and 0.05% solutions in the hot spring water, could inactivate *L. pneumophilla* bacteria to the effective (RF≥3.0 log_{10} CFU/m*l* bacterial reduction) and undetectable level (≤2.6 CFU/m*l*) (RF≥6.35), respectively, within 3 min at 42°C; but in the bath tab water those two solutions could show only appreciable level of efficacy not reaching the undetectable level, yet, at the same contact time. Statistically significant (P<0.05) difference was found between positive control and treatment group (when RF≥3).

DISCUSSION

Disinfectants are used in different fields for the purpose of infectious disease prevention and control [35]. The efficacy of a disinfectant is often tested against laboratory-made bacterial suspensions [8]. In the present study, we tested the efficacy of

disinfectants against laboratory-made *L. pneumophilla* bacterial suspensions after imitating the different field conditions. Different disinfectants are used against *Legionella* for reducing the number of cases and for the prevention of legionellosis outbreaks all over the world [13, 24].

At RT and at 42°C, 200 ppm NaOCl inactivated *L. pneumophilla* to the undetectable level within 5 min in the absence of organic materials, whereas in the presence of organic materials it could not inactivate this bacterium to the undetectable level within 5 min (Tables 1and 2). Hakim *et al.* [20] demonstrated that chlorine solution could inactivate micro-organisms within a very short time, and it was also shown that chlorine can inactivate Newcastle disease virus (NDV) in the air within a short period of time [17]. But, in the presence of organic materials, the inactivation efficacies of the chlorine solution were diminished [4, 17, 49]. Casini *et al.* [10] reported that after years of chlorine treatment, *Legionella* strains were isolated from a water system. Storey *et al.* [44] showed 3 $\log_{10}$ reduction of *L. pneumophila* was achieved after 10 min with a free chlorine concentration of 10 ppm (mg/*l*) at 37°C. Lin *et al.* [24] found that relatively high doses of chlorine (2–6 mg/*l*) were required for continuous control of *Legionella* in water systems, and required much higher doses of chlorine for inactivation of *Legionella* when in association with protozoa.

In the present study, we found that with the presence of organic materials, 0.17% $FdCa(OH)_2$ could inactivate *L. pneumophila* to the undetectable level within 3 min and 5 min, respectively, at 42°C and RT. Toyofuku *et al.* [49] reported that at saturated concentration, 0.17%, $FdCa(OH)_2$ can kill bacteria even in the presence of 5% FBS, while it takes more than 1 min to inactivate them without FBS. $FdCa(OH)_2$ could also inactivate bacteria on egg shell within short time [3]. In the present study we have also found that 0.1 and 0.05% $FdCa(OH)_2$ solutions could inactivate *L. pneumophilla* bacteria >5 log and >4 log reduction, respectively, within 3 min, but 0.025% solution could not inactivate the bacteria effectively within 3 min in the presence of 5% FBS (Table 3). In the present study, we also found that very low concentration of $FdCa(OH)_2$ solution in hot spring water collected from the environment inactivated *L. pneumophilla* bacteria to the effective and undetectable level within 3 min, but in bath water could inactivate only effective level not to the undetectable level (Table 4). Alkaline agents are well-known for their strong bactericidal properties, even in the presence of organic materials [6, 7, 30, 38, 48]. $Ca(OH)_2$ at 0.17% solution is the saturated solution [5, 49], and a higher percentage (>0.17%) of solution worked better than a saturated solution because of containing many nano-particles in the solution [47].

Conversely, Mix200 inactivated *L. pneumophilla* to the undetectable level within 1 and 3 min, respectively, at 42°C and RT, even with the presence organic materials, and effectively within 30 sec and 1 min, respectively (Tables 1 and 2). In our study, we found that the bactericidal efficacy of Mix200 and $FdCa(OH)_2$ solutions was enhanced at higher temperature and required short contact time for the effective inactivation. Recently, we established the synergistic effect of $FdCa(OH)_2$ together with NaOCl for their bactericidal efficacies under the organic material contamination conditions [49], and with QAC for their virucidal efficacies under low temperature conditions [21]. In Japan, heat, ultraviolet, or ozone radiations are used in the 24-hr. home baths for disinfection [http:// 24furo.v.wol.ne.jp/user/03/01.htm].

The mechanism of the synergistic effect appears to be such that high pH and calcium brought about the disruption of bacterial cell membrane [34], thereby allowing OCl to pass through the damaged membrane and denature proteins in the cytoplasm of bacteria, following interaction with proteins and nucleic acids. A similar synergistic mechanism between NaOCl and $FdCa(OH)_2$ was also speculated towards bacterial cell membrane by Toyofuku *et al.* [49]. Ito *et al* [21] demonstrated the similar synergistic efficacies between QAC and $FdCa(OH)_2$ towards avian influenza and Newcastle disease virus. In addition, a similar synergistic bactericidal and virucidal efficacies between QAC and $FdCa(OH)_2$ was confirmed toward *Salmonella* Infantis and *E. coli* on bacteria contaminated abiotic carriers [2] and avian influenza and Newcastle disease virus on virus contaminated abiotic carriers, respectively [1].

In conclusion, our study findings demonstrate that the mixture of NaOCl and $FdCa(OH)_2$ exhibited synergistic effects and showed excellent bactericidal efficacy at both applied temperatures, even in the presence of organic materials. $FdCa(OH)_2$ also showed good bactericidal efficacy at both temperatures in the presence of organic materials, but the bactericidal efficacy of NaOCl became diminished in the presence of organic materials. $FdCa(OH)_2$ solution at 0.2% was evaluated to be in the “non-irritant” category in the primary skin irritation test using rabbits as shown in INTRODUCTION. However, it would be preferable to reduce the concentration of $FdCa(OH)_2$ in bath tab water. It is necessary to replace the water to avoid $Ca(OH)_2$ accumulation. If $FdCa(OH)_2$ powder is added in the concentration of 0.1% into the recycled water that consists of 20% bath tab, it is necessary to completely replace the bath tab water at least every 10 days. It is also important to know that during aeration, $Ca(OH)_2$ will be converted to $CaCO_3$.

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