

Bactericidal Effect of Calcium Oxide and Calcined Shell Calcium on *Legionella pneumophila*

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(Received February 15, 2001; Accepted April 24, 2001)

Both calcined shell calcium, which was recently reported to have bactericidal effects on *Escherichia coli* O157 : H7, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, and calcium oxide, which is the main component in calcined shell calcium, were examined as a bactericide against *Legionella* species. A calcined shell calcium solution of 0.025% or above reduced *L. pneumophila* counts from the original 7.0×10^6 CFU/ml to less than 300 CFU/ml after an incubation period of 1 hr. In water samples taken from the cooling tower of a prefabricated house, a calcined shell calcium solution of 0.05% or above reduced *L. pneumophila* counts to less than 10 CFU/ml after an incubation period of 1 hr. There was no difference between the bactericidal effects of the calcined shell calcium manufactured from the shells of surf clams and that manufactured from oysters; surf clams and oysters are equally effective. Calcium oxide also showed similar bactericidal effects against *L. pneumophila*, and thus we believe that the effect is not specific to calcined shell calcium, but rather to effects caused by the alkalinity of calcium oxide. The use of calcium oxide or calcined shell calcium as a bactericidal agent against *Legionella* species in the cooling tower water of hotels or other buildings is therefore expected.

Key words — *Legionella pneumophila*, cooling tower water, calcined shell calcium, calcium oxide, bactericidal effect

INTRODUCTION

In the natural environment, *Legionella* species exist in the hydrosphere (*i.e.*, moist soil, ponds, marshes, lakes, or rivers). In artificial environments, *Legionella* species have been reported in the cooling tower water of buildings or hotels,¹⁾ water-supply systems or heated-water systems,²⁾ 24-hr circulatory baths,³⁾ humidifiers,⁴⁾ decorative fountains,⁵⁾ and hot spring baths.⁶⁾ The existence of *Legionella* species in a 24-hr circulatory bath caused an outbreak of Legionnaires' disease in a Tokyo home for senior citizens in May 1998. One patient died of pneumonia caused by the *Legionella* species. Cases of infection by *Legionella* species were also reported in water-supply systems or heated-water systems,⁷⁾ the cooling tower water of buildings or hotels,⁸⁾ whirlpool spas,⁹⁾ humidifiers,¹⁰⁾ decorative fountains,¹¹⁾ and hot spring baths.¹²⁾

After these incidents, a counterplan for the prevention of Legionnaires' disease in buildings *etc.* (No. 1679, November 26th, 1999) was prescribed by the Japanese Ministry of Health and Welfare's Environmental Health Bureau. Among the measures prescribed were the disinfection, cleaning, and exchange of water in the cooling towers of buildings or hotels, as well as in water-supply systems, heated-water systems, 24-hr circulatory baths, respirator-humidifiers, decorative fountains, and hot spring baths.

Furthermore, in November 1999, the Building Management Education Center proposed that *Legionella* species must be controlled according to new guidelines to prevent Legionnaires' disease through the use of an infection factor grading system. When inspection for bacteria proves positive, disinfection and cleaning must be carried out to reduce *Legionella* species to an undetectable amount (less than 10 CFU/100 ml). Furthermore, it is required that agents to sterilize *Legionella* species should be infused in cooling tower water to prevent outbreaks of Legionnaires' disease.

At present, although methods such as bactericide by chlorine,¹³⁾ bactericidal agents,¹⁴⁾ and heat, ozone, or UV light¹⁵⁾ have been reported, there is no established method for the sterilization of *Legionella* species. In this study, therefore both calcined shell calcium, which was reported to have bactericidal effects on *Escherichia coli* O157 : H7, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*,^{16,17)} and calcium oxide, which is a main component in calcined shell calcium, were examined as a bactericide

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against *Legionella* species.

MATERIALS AND METHODS

Calcined Shell Calcium and Calcium Oxide — In this study, freely available calcined shell calcium, which is manufactured from surf clams or oysters as a food additive, was used. Calcium oxide of analytical grade was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Determination of *L. pneumophila* Counts — At present, because no official method for the determination of *Legionella* species counts exists in Japan,¹⁸⁾ the culture method using buffered charcoal-yeast extract (BCYE α) agar medium (used widely for the determination of *L. pneumophila* counts) was adopted in this study. The test organism was *L. pneumophila* serogroup 1 taken from cooling tower water.

Evaluation of Bactericidal Effects —

1. Calcined shell calcium solutions and calcium oxide solutions of 0, 0.005, 0.025, 0.05, 0.1, 0.5, and 1.0 w/v % were prepared with 200 ml of distilled water. In the same way, 0, 0.005, 0.025, 0.05, 0.1, and 0.5 w/v% calcined shell calcium solutions and calcium oxide solutions were prepared with 100 ml of cooling water taken from a prefabricated house.
2. The test organisms were added to physiological saline to make a test suspension of McFarland 5.
3. One milliliter of the test suspension prepared at stage 2 was added to the 200 ml (100 ml for cooling tower water) of each calcined shell calcium solution and calcium oxide solution.
4. After the addition of the test suspension, both the calcined shell calcium solution and calcium oxide solution were stirred well. After the addition of the test suspension, each solution was incubated at room temperature and samples were taken after intervals of 1, 24, and 48 hr. Serial dilutions of the sample were made and 0.1 ml of each dilution was spread on agar plates. In the case of the 0% calcined shell calcium solution and calcium oxide solution, sampling also was carried out before incubation.
5. Colonies of *L. pneumophila* were counted after incubation periods of 7 days. The original counts were determined from the serial dilutions of 0% solutions before incubation.

Table 1. Bactericidal Effects of Calcined Shell Calcium Solution against *L. pneumophila*

Sample concentration %	<i>L. pneumophila</i> count (CFU/ml)		
	After 1 hr	After 24 hr	After 48 hr
1.000	< 300	< 300	< 300
0.500	< 300	< 300	< 300
0.100	< 300	< 300	< 300
0.050	< 300	< 300	< 300
0.025	< 300	< 300	< 300
0.005	41000	< 300	< 300

Time 0: 7000000 CFU/ml

Table 2. Bactericidal Effects against *L. pneumophila* of Calcined Shell Calcium Prepared with Stabilized Cooling Tower Water

Sample concentration %	<i>L. pneumophila</i> count (CFU/ml)			
	0 time	After 1 hr	After 24 hr	After 48 hr
0	7.9×10^5	7.7×10^5	8.7×10^5	7.2×10^5
0.005	7.9×10^5	6.7×10^5	3.0×10^5	2.2×10^5
0.025	7.9×10^5	< 10	< 10	< 10
0.050	7.9×10^5	< 10	< 10	< 10
0.100	7.9×10^5	< 10	< 10	< 10

RESULTS

Bactericidal Effects of Calcined Shell Calcium Solution against *L. pneumophila* (Table 1)

A calcined shell calcium solution of 0.025% or above reduced *L. pneumophila* counts from the original 7.0×10^6 CFU/ml to less than 300 CFU/ml after an incubation period of 1 hr. *L. pneumophila* counts did not increase after incubation periods of 24 or 48 hr. A 0.005% solution reduced counts to about 1/100 of the original counts after an incubation period of 1 hr and to less than 300 CFU/ml after an incubation period of 24 hr. *L. pneumophila* counts did not increase after an incubation period of 48 hr.

Bactericidal Effects of Calcined Shell Calcium Prepared with Cooling Tower Water against *L. pneumophila*

Bactericidal effects were tested in cooling tower water taken from a prefabricated house. The calcined shell calcium was prepared in both sterilized and nonsterilized cooling tower water, because it was expected that various bacteria were present.

Table 2 shows the bactericidal effects against *L. pneumophila* of calcined shell calcium prepared with

sterilized cooling tower water. In sterilized cooling tower water, a calcined shell calcium solution of 0.025% or above reduced *L. pneumophila* counts from the original 7.9×10^5 CFU/ml to less than 10 CFU/ml after an incubation period of 1 hr. *L. pneumophila* counts did not increase after incubation periods of 24 or 48 hr.

A 0.005% solution resulted in a slight decrease in *L. pneumophila* counts, and this decrease was therefore not attributable to bactericidal effects. In the control (0%) solution, few changes were observed at all intervals up to 48 hr, and the changes were therefore not attributable to bacterial effects.

Table 3 shows the bactericidal effects against *L. pneumophila* of calcined shell calcium prepared with nonsterilized cooling tower water. The bacterial counts in nonsterilized cooling tower water were as follows: *Legionella* counts 4400 CFU/100 ml; total counts of microorganisms were 51 CFU/ml; and coliform was not detected. In nonsterilized cooling water, a calcined shell calcium solution of 0.05% or above reduced *L. pneumophila* counts from the original 8.2×10^5 CFU/ml to less than 10 CFU/ml after an incubation period of 1 hr. *L. pneumophila* counts did not increase after incubation periods of 24 or 48 hr. A 0.025% solution reduced *L. pneumophila* counts to about 1/100 of the original counts after an

incubation period of 1 hr and to less than 10 CFU/ml after 24 hr. *L. pneumophila* counts did not increase after an incubation period of 48 hr. A 0.005% solution resulted in only a slight decrease in *L. pneumophila* counts, and this decrease was therefore not attributable to bactericidal effects. In the control (0%) solution, few changes were observed at all intervals up to 48 hr, and these changes were therefore not attributable to bactericidal effects.

Comparison of the Bactericidal Effects of Calcium Oxide and Those of Calcined Shell Calcium

Because a main component of calcined shell calcium is calcium oxide, the bactericidal effects of calcium oxide against *L. pneumophila* were tested. Calcined shell calcium prepared from surf clams or from oysters was also tested. The results are shown in Table 4. A 0.005% calcined shell calcium solution both from surf clams and from oysters reduced *L. pneumophila* counts to 5700 CFU/ml and 5300 CFU/ml, respectively, after an incubation period of 1 hr and to less than 10 CFU/ml from the original 4.3×10^5 CFU/ml after an incubation period of 24 hr. *L. pneumophila* counts did not increase after an incubation period of 48 hr. Similarly, a 0.005% calcium oxide solution reduced *L. pneumophila* counts from the original 4.3×10^5 CFU/ml to 5000 CFU/ml after an incubation period of 1 hr and to less than 10 CFU/ml after an incubation period of 24 hr. *L. pneumophila* counts did not increase after an incubation period of 48 hr.

Table 3. Bactericidal Effects against *L. pneumophila* of Calcined Shell Calcium Prepared with Nonsterilized Cooling Tower Water

Sample concentration %	<i>L. pneumophila</i> (CFU/ml)			
	0 time	After 1 hr	After 24 hr	After 48 hr
0	8.2×10^5	1.3×10^6	7.3×10^5	6.0×10^5
0.005	8.2×10^5	1.1×10^6	5.5×10^5	4.6×10^5
0.025	8.2×10^5	7.9×10^4	< 10	< 10
0.050	8.2×10^5	< 10	< 10	< 10
0.100	8.2×10^5	< 10	< 10	< 10

DISCUSSION

In the experiments on the bactericidal effects against *L. pneumophila*, 0.025% calcined shell calcium was able to reduce *L. pneumophila* counts by four digits, and the bactericidal effects of calcined shell calcium against *L. pneumophila* was thus con-

Table 4. Comparison of the Bactericidal Effects of Calcium Oxide and Those of Calcined Shell Calcium

Sample concentration %	<i>L. pneumophila</i> count (CFU/ml)								
	Surf clam			Oyster			Calcium oxide		
	After 1 hr	After 24 hr	After 48 hr	After 1 hr	After 24 hr	After 48 hr	After 1 hr	After 24 hr	After 48 hr
0	490000	240000	200000	490000	240000	200000	490000	240000	200000
0.100	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
0.050	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
0.025	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
0.005	5700	< 10	< 10	5300	< 10	< 10	5000	< 10	< 10

L. pneumophila counts at time 0: 430000 CFU/ml.

firmed. Because a 0.025% calcined shell calcium solution has a strong alkalinity (pH 12),¹⁷⁾ *Legionella* species that mainly grow at pH of 6.8–6.9⁵⁾ cannot exist. In experiments utilizing cooling tower water from a prefabricated house, *L. pneumophila* counts were 4400 CFU/100 ml. This is equivalent to the 10²–10⁴ CFU/100 ml *Legionella* species counts reported¹⁾ in cooling tower water containing no chemical agents. Calcined shell calcium solution levels of 0.025% can reduce *L. pneumophila* counts to less than 10 CFU/ml after an incubation period of only 1 hr, and these bactericidal effects were confirmed in actual cooling tower water. Even if bacteria other than *L. pneumophila* existed in the nonsterilized cooling tower water, sufficient bactericidal effects were confirmed in the 0.05% calcined shell calcium solution. This effect was comparable to the effects of sodium hypochlorite solution, which corresponds to a level of free residual chlorine of 0.4 mg/l.¹³⁾ Taking into account the new governmental guidelines (*i.e.*, when a test for *Legionella* species proves positive, the cooling tower must be cleaned and disinfected and *Legionella* species counts must be reduced to an undetectable amount [10 CFU/100 ml] to prevent Legionnaires' disease) as proposed by the Building Management Education Center, we consider calcined shell calcium useful as a bactericidal agent against *L. pneumophila* in cooling tower water. Calcium oxide, which is known to be a disinfectant, showed similar bactericidal effects against *L. pneumophila*. It can therefore be concluded that the bactericidal effects were not specific to calcined shell calcium but rather result from the alkaline effects of calcium oxide. Furthermore, the shells of both surf clams and oysters have similar bactericidal effects, so it can be concluded that there is no difference in the bactericidal effects of either type of shell. Sufficient bactericidal effects of both calcined shell calcium and calcium oxide were confirmed against *Legionella* species in actual cooling tower water. Therefore we concluded that these calcium preparations could be of practical use in sterilizing *Legionella* species in cooling towers.

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