Note

Inactivation of *Legionella pneumophila* from Whirlpool Bath Waters by Grapefruit (*Citrus paradisi*) Seed Extract

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We investigated the antibacterial effect of commercial grapefruit seed extract (GSE, Liquid), which has recently been attracting attention as a food additive, on *Legionella pneumophila*. The sensitivity test was performed using the disc method. All 25 strains tested were sensitive to GSE, and formed clear inhibition zones in its presence. The minimum inhibitory concentration (MIC) values were measured by the agar dilution method. The MIC of GSE in the strains was in the range of 98mg/l to 390mg/l, and the MIC₉₀ was 390mg/l. The minimum bactericidal concentration (MBC) values for the test strains were also determined. MBC₉₀ was 50,000, 6,300, and 3,100mg/l when the exposure time was 1, 10, and 60 minutes, respectively. These bactericidal test results demonstrate that GSE has a bactericidal effect on *L. pneumophila*.

Key words : Grapefruit seed extract/*Legionella pneumophila*/Bactericidal activity.

Legionellosis caused by bacteria of the *Legionella* species has been attracting attention as a serious public health problem in recent years in Japan. In July 2002, an outbreak causing seven deaths and 295 cases of infection occurred in a special public bath in Hyuga City, Miyazaki (National Institute of Infectious Diseases, 2003). An epidemiological investigation revealed that the source of infection was the circulating bath water, indicating the importance of countermeasures against this source of infection. In public baths, where such infections have frequently been reported, sanitary conditions are maintained by using chlorine disinfection, but sufficient disinfection cannot be expected when the pH of the bath water is high or there is a high concentration of organic compounds. Moreover, additional equipment used in public baths such as for aeration may markedly decrease the effectiveness of the disinfection. Based on these considerations, we have investigated natural antibacterial agents as substitutes for chlorine disinfection, and re-

ported the effects of coffee (Furuhata et al., 2000a and 2002) and herbs (Furuhata et al., 2000b) on *L. pneumophila*.

In this study, we investigated the antibacterial effect of grapefruit (*Citrus paradisi*) seed extract (GSE), which has recently attracted attention in the food industry field as an agent that prolongs preservation.

Commercial GSE (CAP-10, Liquid, Kalfachemical Co., Kanagawa) was used. Twenty-six bacterial strains were used: *L. pneumophila* serogroup 1 IID5232 strain (No. 26) and four strains of *L. pneumophila* serogroup 1 (No. 1–4), five strains of serogroup 3 (No. 5–9), two strains of serogroup 4 (No. 10, 11), nine strains of serogroup 5 (No. 12–20), and five strains of serogroup 6 (No. 21–25) isolated from whirlpool bath waters in metropolitan areas.

The GSE was sterilized by filtration using a disposable membrane filter unit with a pore size of 0.20 µm and a diameter of 25mm (Dismic-25cs, Advantec Toyo Co., Tokyo). Fifty microliters of the sterilized GSE were slowly adsorbed to a paper disc for the sensitivity test (diameter: 8 mm, thickness: 1.5mm, Advantec Toyo Co., Tokyo).

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The bacterial strains were cultured on BCYE α agar medium (Nikken Biomedical Laboratory Co., Kyoto) at 37°C for 3 days, and individual bacterial colonies were suspended in sterilized distilled water, and adjusted to a turbidity corresponding to McFarland No.1 (10^8 cfu/ml). A 0.1-ml aliquot of this bacterial suspension was inoculated onto B-SYE agar medium (Sawatari et al., 1984) (Nikken Biomedical Laboratory Co., Kyoto). After the bacterial suspension had sufficiently been adsorbed, the antibacterial test discs were allowed to adhere to the surface of the medium and the plates were cultured at 37°C for 7 days. After the culture period, the inhibition zone around each disc was examined and its diameter was measured.

The MIC was measured according to the agar plate dilution method (NCCLS, 2000), which is the method used to measure the MIC of antibiotics. The test GSE was subjected to two-fold serial dilutions, and 2ml of each dilution were added to dishes and combined with 18ml of B-SYE agar medium (Sawatari et al., 1984) to prepare drug plates. Bacterial suspensions were prepared as described above, and 5 μ l of the suspension were inoculated onto each drug plate using a Microplanter MIT-P (Sakuma Seisakusho Co., Tokyo). These plates were incubated at 37°C for 7 days. The presence or absence of growth of the tested strains at each concentration, and the minimum inhibitory concentration (MIC) were determined.

To each well of a flat-bottom 96-well microplate (Greiner Japan Co., Tokyo) 100 μ l of sterilized water was added, and mixed with GSE (initially diluted 10-fold) to prepare serial two-fold dilutions. To these wells, 10 μ l of the bacterial suspension prepared as described above was added and kept at room temperature for 1, 10, or 60min. At each of these time points, a 10- μ l aliquot was collected from each well and spotted on an BCYE α agar plate (Nikken Biomedical Laboratory Co., Kyoto). After the BCYE α plates were incubated at 37°C for 7 days, the formation of colonies from these spots was observed and the minimum concentration of GSE resulting in no growth of the test bacteria was determined as the minimum bactericidal concentration (MBC).

The tested GSE was added to 200ml of phosphate buffer pH 7.0 kept at 40°C to prepare 1,000-fold (1,000mg/l) and 10,000-fold (100mg/l) dilutions. *L. pneumophila* IID 5232 strain (No.26) or bath-water-derived *L. pneumophila* serogroup 5 (No.12) was inoculated in the solutions at 10^6 cfu/ml, and kept at 40°C. The solutions were sampled at 1, 3, and 5 minutes, at which times 0.1ml of each solution was immediately smeared on BCYE α agar (Nikken Biomedical Laboratory Co. Kyoto). The plates were

incubated at 37°C for 7 days and the viable cells were counted.

The same experiment was performed using a buffer solution adjusted to pH 9.0. In addition, to examine the ability of GSE to disinfect medicated bath water, phosphate buffer (pH 7.0) containing 1% of a crude drug taken as a model of medicated bath water (Yakujinto, containing Phellodendri Cortex, Angelicae Radix, Foeniculi Fructus, Zingiberis Rhizoma, Cnidii Rhizoma, Paeoniae Radix, Aurantii Nobilis Pericarpium, and Pepper; Morishita Jintan Co., Osaka) was prepared, and combined with the same volumes of the test samples of GSE as used in the bactericidal test. For controls, buffer solutions without the addition of GSE were used.

The sizes of the inhibition zones of GSE for the test strains were measured. Clear inhibition zones were observed for all test strains (25 strains), and the diameter varied from 17mm to 25mm, with a mean $\pm$ standard deviation of 20.2 ± 2.4 mm, without significant difference among the test strains. The mean size of the inhibition zone for each of serogroups 1, 3, 4, 5, 6 varied from 20mm to 22mm, without significant difference depending on the serogroup. Therefore, GSE exerted antibacterial effect on *L. pneumophila*.

The cumulative fractions of the tested bacterial strains inhibited at each MIC value are presented in the order from lower to higher concentrations in Figure 1 to show the distribution range of MIC. The MIC ranged from 98mg/l to 390mg/l depending on the strain, with the minimal growth inhibition distributed within three sequential tubes (eight-fold). The MIC values for 50% and 90% of the strains (MIC_{50} and MIC_{90} , respectively) were both 390mg/l.

FIG. 1. Cumulative distribution of MIC of the grapefruit seed extract against *L. pneumophila* from whirlpool bath waters.

The cumulative distribution of MBC at each time point of exposure was examined in a manner similar to that for MIC. As the duration of exposure increased, the MBC distribution pattern shifted to the left, showing that the bacteria tended to be killed at lower concentrations. MBC ranged from 13,000 to 50,000mg/l with an exposure of 1 min, while it decreased to a range from 1,600 to 6,300mg/l with an exposure of 10min and to a range of 780 to 3,100mg/l with an exposure of 60min. At all exposure times, MBC was seen in samples distributed within three tubes (an eight-fold dilution range), showing a similar distribution range as MIC. The MBC₅₀ was 25,000, 3,100, and 1,600 mg/l at 1, 10, and 60 min of exposure time, respectively, and MBC₉₀ was 50,000, 6,300, and 3,100 mg/l, respectively (Table 1).

Changes in the *L. pneumophila* cell count caused by incubation in phosphate buffers at pH 7.0 and pH 9.0 containing 100mg/l GSE and 1,000mg/l GSE, and in phosphate buffer pH 7.0 containing the crude drug (Yakujinto) were measured. Under all conditions, the initial inoculum size, 10⁶cfu/ml, was decreased by 4 log cycles (or more than 99.99%) within 1 min. Particularly, the number of inoculated organisms decreased promptly even when the Yakujinto being used as the medicated bath water was added. The decreases found for the standard strain IID 5232 and the isolates from bath water showed no significant difference.

GSE has recently been attracting attention as a natural antibacterial agent, and it has been shown to have a wide antibacterial spectrum (Cho et al., 1995 ; Ionescu et al., 1990). The antibacterial activity of GSE is considered to make it an effective agent for extending the storage period of food in Japan, and its safety has been confirmed (Watanabe and Yamaguchi, 1995). It has been found that the antibacterial activity of GSE is mainly due to the component benzetonium chloride (Takeoka et al., 2001 ; von Woedtke et al., 1999). With regard to the mechanism of action, this substance has been found to cause the rupture of the bacterial membrane and release of the cytoplasm (Heggers et al., 2002).

TABLE 1. MBC₅₀, MBC₉₀ and MBC ranges of grapefruit seed extract against *L.pneumophila* from whirlpool bath waters.

Contact time (min)	MBC (mg/l)		
	Range	MBC ₅₀	MBC ₉₀
1	13,000-50,000	25,000	50,000
10	1,600-6,300	3,100	6,300
60	780-3,100	1,600	3,100

n=25

In this study, we investigated the antibacterial effect of GSE on the etiologic agent of legionellosis, *L. pneumophila*. In screening tests using the disc method, all test strains formed inhibition zones, showing an obvious antibacterial effect of GSE. The size of the inhibition zones did not significantly differ among the strains, and similar degrees of growth inhibition were observed. Although there have been many reports on the antimicrobial effects of GSE on various microorganisms (Heggers et al., 2002 ; Reagor et al., 2002), to our knowledge no data on *L. pneumophila* have hitherto been reported.

Nishina et al. (1991) reported that the MIC values of GSE for *E. coli* and *S. aureus* were 25 and 5mg/l, respectively, indicating that GSE has potent antibacterial activity. Watanabe et al. (1995) reported the antibacterial activity of another type of GSE, with MIC values for *E. coli* and *S. aureus* of 200 and 25 mg/l, respectively.

In this study, we found that the MIC value of GSE for *L. pneumophila* (MIC₉₀) was 390mg/ml, which is lower than those of coffee-derived caffeic acid and chlorogenic acid, both at 630mg/l (Furuhata et al., 2002), showing the high antibacterial activity of GSE. In addition, the MBC₉₀ of GSE at an exposure time of 60 min was 3,100mg/l, indicating that GSE has higher bactericidal activity than the coffee-derived substances. Investigation of the time-course of the changes in the viable bacterial count in the bactericidal test revealed that 100mg/l GSE killed 99.99% or more of the bacteria within 1 min. These findings suggest that GSE is an effective natural bactericide for *L. pneumophila*.

Takeoka et al. (2001) identified the bactericidal component of commercial GSE as benzetonium chloride. In the present study, though we did not identify the antibacterial material, it is possible that the same component as that identified by Takeoka et al. was responsible for the bactericidal effect.

Disinfection with chlorine products is generally recommended for hot springs and public baths shown to be contaminated with *Legionella* spp. in Japan, and simple chlorination at a high concentration is performed in some places. Water characteristics such as pH vary markedly among hot springs, and there are various kinds of public baths, including medicated baths. In such circumstances, the control of *Legionella* spp. by chlorination alone is very difficult. The results of this study suggest that the bactericidal effect of GSE does not decrease at pH 9.0 or in medicated baths (for example, medicated with Yakujinto), and that GSE would therefore be useful as a bactericide for practical use. This point will require verification in tests during actual use.

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