Effects of Japan Sea Proper Water on the Growth of *Legionella pneumophila*, *Escherichia coli*, and *Staphylococcus aureus*

Yasuo TSUCHIYA¹, Michihiro TERAO², Takanori FUJIMOTO¹, Kazutoshi NAKAMURA¹ and Masaharu YAMAMOTO¹

¹Department of Community Preventive Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
²Department of Medical Technology, School of Health Sciences Faculty of Medicine, Niigata University, Niigata, Japan

Abstract

Objective: To assess whether *Legionella pneumophila* serogroup 1 and serogroup 6, *Escherichia coli*, and *Staphylococcus aureus* can survive in Japan Sea Proper Water (JSPW).

Methods: The inhibitory effects of JSPW, surface seawater (SSW), phosphate buffer solution with 3.5% NaCl of pH 7.0 (3.5%NaClPBS), and the 10² and 10⁴-fold dilute solutions with purified water or phosphate buffer solution of pH 7.0, and purified water were investigated. Survival cells were counted immediately after the water and the bacteria were mixed, and at 1, 3, 5, and 7 days after incubation at 37°C. If the number of surviving cells was decreased more than 2 log units compared with the starting value, we judged the medium to have had an inhibitory effect on the growth of the bacteria.

Results: The survival cells of the bacteria in JSPW had decreased more than 2 log units compared with the starting value at 1 day after incubation. After 1 day of incubation, the cells of *Legionella pneumophila* serogroup 6 and *Staphylococcus aureus* were found to have decreased more than 2 log units in purified water (PW) used as a control. Furthermore, *Legionella pneumophila* serogroup 1 in the 10²-fold dilute solution of JSPW was only 1.04 log units lower than the starting value at 7 days after incubation. In the 10² and 10⁴-fold dilute solutions of JSPW, *Escherichia coli* survived for 7 days after incubation. These results were almost similar to the results in SSW and 3.5%NaClPBS.

Conclusions: The present findings demonstrate that *Legionella pneumophila* serogroup 1 and *Escherichia coli* cannot survive in undiluted JSPW for over a day at 37°C, suggesting the inhibitory effects may be due to the sodium chloride contained in JSPW.

Key words: bath water, inhibitory effect, surface seawater, *Legionella pneumophila*, *Escherichia coli*, *Staphylococcus aureus*

Introduction

Japan Sea Proper Water (JSPW) has been collected from the Sado offing of the Japan Sea of Niigata Prefecture as a new resource. Recently, the use of JSPW in thalassotherapy has begun. Japanese people traditionally make a practice of daily bathing and may prefer to bathe in hot springs, getting away whenever they can for a relaxing stay at their favorite resorts. In general, hot spring bathing is useful for maintaining hygiene, improving blood circulation, and mitigating muscular fatigue and pain because of the water's warm temperature, pressure, and buoyancy effect (1). In addition, it has been demonstrated that the salt in hot spring water has the effect of raising body temperature for a longer period. Previous studies regarding the effects of balneotherapy in the treatment of some diseases at the Dead Sea have been published (2–5). Furthermore, we previously demonstrated that hot JSPW bathing had a beneficial influence on human health (6, 7). Therefore, it is expected that use of JSPW will be promoted for human health.

Many bacteria exist in our environment. Of these bacteria, *Legionella pneumophila* (*L. pneumophila*) has been detected in environmental water and soil, and Legionnaires' disease, which is caused by the bacteria, is a growing public health concern. *L. pneumophila* can survive under a wide range of environmental conditions: temperatures from 0°C to 63°C; pH from 5.0 to 8.5; dissolved oxygen level from 0.2 to 15.0 mg/L (8). When
JSPW is used as bath water, the water is contaminated, after people have bathed in it, with not only L. pneumophila but also Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus). E. coli is isolated from human feces, and S. aureus is one of the indigenous microbiota on human skin. Therefore, it is important to determine the antibacterial activities of JSPW that inhibit the growth of the bacteria in order to prevent the infections that may be caused by taking a JSPW bath.

Previously, several authors have reported that Legionella species can survive in sea water (9–11). Nevertheless, some trials demonstrated an inhibitory effect of sodium chloride (NaCl) on growth of Legionella species (12–14). Thus, previous reports on inhibitory effects of sea water on growth of Legionella species have been conflicting.

Because JSPW contains about 3.5% NaCl (15), a lot of bacteria may not be capable of growing in JSPW. However, to our knowledge, no study has investigated the effect of JSPW on the survival of bacteria.

The aim of the present study was to determine the ability of L. pneumophilia, E. coli, and S. aureus to survive in JSPW. The results of JSPW were compared with those of surface seawater (SSW) and other media including phosphate buffer solution with 3.5% NaCl of pH 7.0 (3.5% NaCl PBS), phosphate buffer solution of pH 7.0 (PBS), and purified water (PW).

**Materials and Methods**

1. **Collection of JSPW and SSW**

   JSPW and SSW were collected from depths of about 300 meters and 0 meters in the Sea of Japan (37.50–38.00°FN latitude; 138.30–138.45°E longitude), respectively, using the JSPW-drawing system developed jointly by HONMA Corporation (Niigata, Japan) and KITAC Corporation (Niigata). Water was collected in a one-liter plastic container and sent immediately, under refrigeration, to our laboratory. The water was stored in a refrigerator and used within 7 days.

2. **Bacterial strains**

   Two strains of L. pneumophilia were used in this study. One strain (serogroup 1; SG1) was obtained from Denka Seiken Co., Ltd. (Tokyo, Japan). The other (serogroup 6, strain 25K; SG6) was isolated from environmental water and identified. E. coli (ID 5208) and S. aureus (ID 980) were also used in this study.

3. **Preparation of bacteria**

   To obtain inocula for the test, L. pneumophilia SG1 and SG6 were cultured on glycine-vancomycin-polyomixin B-amphotericin B supplemented with α-ketoglutarate (WYOa, Eiken Chemical Co. Ltd., Tokyo) agar, which is a selecting agar for Legionella species, for 5 days at 37°C. E. coli and S. aureus were cultured overnight on Nutrient agar medium (Eiken Chemicals Co. Ltd.) at 37°C. After the incubation, each bacteria suspension was prepared in sterilized purified water, and the concentration of the bacteria was adjusted to approximately 10⁴ colony-forming units per milliliter (cfu/ml).

4. **Surviving cell count**

   To assess the inhibitory effects of JSPW on the growth of L. pneumophilia, E. coli, and S. aureus, bacteria suspensions that were 0.5 ml in volume were added to 4.5 ml of each of the following 11 kinds of water: JSPW, SSW, 3.5% NaCl PBS, and their 10⁻³- or 10⁻⁵-fold dilute solutions with PW, PBS, and PW, respectively. PBS was purchased from Wako Pure Chemicals, Co. Ltd. (Osaka, Japan), and PW was made using Millipore ELIX 5 (Japan Millipore Corporation, Tokyo). All the testing water samples were sterilized by being passed through a 0.45 μm syringe filter (Milex-HV, Millipore Corporation, Tokyo, Japan).

   Immediately after the water and the bacteria suspension were mixed, each 0.1 ml of 2 Legionella suspensions was inoculated on WYOa and buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutarate (BCYEa) agars. Then the bacteria were incubated for 5 days at 37°C. Meanwhile, each 0.1 ml of the suspensions of E. coli and S. aureus were inoculated on Nutrient agar medium, and the medium was stored overnight in an incubator at 37°C. Two plates per one water sample were used in this study. After the incubation, the surviving cells on the agars were counted and then the mean value of the cells on duplicate plates was calculated. The value was used as the starting value in each water sample.

   The remaining suspension was incubated at 37°C in a shaking incubator operating at 50 times per min. After incubation for 1, 3, 5, and 7 days, the bacteria were incubated by the methods described above, and the mean values of the surviving cells were calculated.

   We examined the inhibitory effects of JSPW and PW on the growth of L. pneumophilia SG1 and SG6 in 3 different concentrations of bacteria: 2×10² (Concentration 1), 4×10⁴ (Concentration 2), and 4×10⁶ (Concentration 3) cfu/ml. Each bacteria suspension was added to JSPW and PW, and the mixtures were incubated in a shaking incubator for 1 day at 37°C. Both before and after incubation, the bacteria were inoculated on BCYEa agars and incubated for 5 days at 37°C. After 1 day of incubation, the surviving cells on the agars were counted.

   We judged that the water had an inhibitory effect on the growth of the bacteria when the number of surviving cells was decreased more than 2 log units compared with the starting value.

**Results**

1. **Inhibitory effects of the tested water samples on the growth of L. pneumophilia**

   L. pneumophilia SG1 and SG6 were used to assess the inhibitory effects of JSPW, SSW, 3.5% NaCl PBS, PBS, and PW. The experiment was performed 5 times within 7 days (i.e., immediately after the mixture of the water and bacteria, and at 1, 3, 5, and 7 days after incubation), and the surviving cells were counted. As shown in Table 1, the starting value of L. pneumophilia SG1 on BCYEa agar was between 3.94 and 5.43 log units. After 1 day of incubation on BCYEa agar, the number of surviving cells in JSPW, SSW, 3.5% NaCl PBS, and
The 10^6-fold dilute solution of 3.5% NaCl PBS had decreased more than 2 log units compared with the starting value. In the 10^4-fold dilute solutions of SSW and 3.5% NaCl PBS, and the 10^3-fold dilute solutions of JSPW and SSW, the cells had decreased more than 2 log units compared with the starting value at 3 days after incubation. Additionally, the cells in PW had decreased more than 2 log units compared with the starting value at 5 days after incubation. The cell numbers in the 10^2-fold dilute solution of JSPW or PBS were 1.04 or 1.39 log units lower at 7 days after incubation than the starting value. The changes in the number of surviving cells on WYOα agar were quite similar to the results obtained on BCYEα agar.

Table 2 shows the inhibitory effects of the tested water samples on the growth of L. pneumophila SG6. The starting value was between 3.45 and 4.43 log units on BCYEα agar. After 1 day of incubation on BCYEα agar, the number of surviving cells in all tested water samples except for the 10^2-fold dilute solution of 3.5% NaCl PBS and PW had decreased more than 2 log units from the starting values. In the 10^3-fold dilute solution of 3.5% NaCl PBS, the cell number was 1.78 log units lower at 1 day after incubation than the starting value. In PW, the cell numbers had decreased more than 2 log units at 5 days after incubation than the starting value. Although the starting values on WYOα agar were slightly lower than those on BCYEα agar, the changes in the numbers of surviving cells after incubation showed tendencies quite similar to those on BCYEα agar.

We also examined the inhibitory effects of JSPW and PW on the growth of L. pneumophila SG1 and SG6 in the different bacteria concentrations. In JSPW, the cells of L. pneumophila SG1 and SG6 in concentrations 1 and 2 had decreased more than 2 log units at 1 day after incubation. In PW, however, the cells of L. pneumophila SG1 and SG6 had decreased more than 2 log units at 1 day after only in concentration 1. Inhibitory effects of JSPW on the growth of L. pneumophila SG1 and SG6 in concentrations 1 and 2 were found at 1 day after incubation.
2. Inhibitory effects of the tested water samples on the growth of E. coli and S. aureus

The inhibitory effects of the tested water samples on the growth of E. coli and S. aureus are shown at the top and bottom of Table 3, respectively. The starting values of E. coli were between 4.46 and 4.59 log units. E. coli in JSPW and SSW had decreased more than 2 log units at 1 day after incubation than the starting value. In 3.5%NaCIPBS, the cell numbers had decreased more than 2 log units at 3 days after incubation compared with the starting value. However, E. coli in the 10^2- and 10^4-fold dilute solutions of JSPW, SSW, and 3.5% NaCIPBS, and that in PBS and PW, survived for 7 days after incubation. On the other hand, the starting values of S. aureus were between 4.63 and 4.94 log units, and the cell numbers in all of the tested water samples had decreased more than 2 log units compared with the starting value at 1 day after incubation. S. aureus could not survive 3 days after incubation in any of the tested water samples.

Table 3 Survival of Escherichia coli and Staphylococcus aureus in water samples

(a) Escherichia coli

<table>
<thead>
<tr>
<th>Testing water samples</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>JSPW</td>
<td>4.46</td>
</tr>
<tr>
<td>JSPWx10^-2</td>
<td>4.48</td>
</tr>
<tr>
<td>JSPWx10^-4</td>
<td>4.56</td>
</tr>
<tr>
<td>SSW</td>
<td>4.48</td>
</tr>
<tr>
<td>SSWx10^-2</td>
<td>4.59</td>
</tr>
<tr>
<td>SSWx10^-4</td>
<td>4.48</td>
</tr>
<tr>
<td>3.5%NaCIPBS</td>
<td>4.53</td>
</tr>
<tr>
<td>3.5%NaCIPBSx10^-2</td>
<td>4.49</td>
</tr>
<tr>
<td>3.5%NaCIPBSx10^-4</td>
<td>4.57</td>
</tr>
<tr>
<td>Purified water</td>
<td>4.52</td>
</tr>
<tr>
<td>PBS</td>
<td>4.54</td>
</tr>
</tbody>
</table>

(b) Staphylococcus aureus

<table>
<thead>
<tr>
<th>Testing water samples</th>
<th>Incubation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>JSPW</td>
<td>4.69</td>
</tr>
<tr>
<td>JSPWx10^-2</td>
<td>4.79</td>
</tr>
<tr>
<td>JSPWx10^-4</td>
<td>4.90</td>
</tr>
<tr>
<td>SSW</td>
<td>4.86</td>
</tr>
<tr>
<td>SSWx10^-2</td>
<td>4.91</td>
</tr>
<tr>
<td>SSWx10^-4</td>
<td>4.93</td>
</tr>
<tr>
<td>3.5%NaCIPBS</td>
<td>4.85</td>
</tr>
<tr>
<td>3.5%NaCIPBSx10^-2</td>
<td>4.71</td>
</tr>
<tr>
<td>3.5%NaCIPBSx10^-4</td>
<td>4.63</td>
</tr>
<tr>
<td>Purified water</td>
<td>4.73</td>
</tr>
<tr>
<td>PBS</td>
<td>4.94</td>
</tr>
</tbody>
</table>

JSPW: Japan Sea Proper Water, SSW; surface seawater, 3.5%NaCIPBS; phosphate buffer solution with 3.5% NaCl of pH 7.0, PBS; phosphate buffer solution of pH 7.0. DSW, SSW, and 3.5%NaCIPBS were diluted with purified water to 10^-1- and 10^-4-fold dilute solutions. Values show logarithmic colony forming unit per milliliter (log cfu/ml) after incubation at 37°C.

Discussion

In the present study, we found that the surviving cells of L. pneumophila SG1 and SG6, E. coli, and S. aureus had decreased more than 2 log units in undiluted JSPW at 1 day after incubation at 37°C. In the control PW, however, the surviving cells of L. pneumophila SG6 and S. aureus had decreased more than 2 log units at 1 day after incubation, as in JSPW. This provides evidence that undiluted JSPW has ability to inhibit the growth of L. pneumophila SG1 and E. coli under the present experimental conditions.

Before making efficient use of JSPW as bath water, it is very important to assess the inhibitory effects of JSPW on the growth of bacteria in order to prevent microbial infections that can be caused by JSPW bathing. L. pneumophila is a facultative bacterium, and Legionnaires’ disease is normally caused by the inhalation or aspiration of Legionella from contaminated aquatic bodies including rivers, lakes, streams, and thermally polluted waters (8). Legionella usually does not harm healthy people, but can seriously infect immunocompromised patients in hospitals or elderly people. Legionnaires’ disease has been recognized in many countries throughout the world (16-19). In addition, a large outbreak of Legionnaires’ disease caused by an inadequate circulating and filtration system for communal bath water has been reported in Japan (20). In bathhouses or hot springs used by the general public, it is necessary to confirm not only the extent of bacterial contaminations such as L. pneumophila, E. coli, or S. aureus but also the inhibitory effects of bath water on the growth of the bacteria for the purpose of preventing transmission. We conducted this study to clarify whether JSPW has an inhibitory effect on the growth of L. pneumophila SG1 and SG6, E. coli, or S. aureus.

The activities of bacteria are greatly affected by the chemical and physical conditions of their environment, such as temperature, pH,ionic strength or osmosis, oxygen, carbon dioxide, and humidity. Although temperature is one of the most important environmental factors influencing the growth and survival of bacteria, pH and osmosis are also important factors. Because each bacterium usually has a well-defined optimum pH for growth (near the neutral pH range, pH 6-7.5), we used a pH buffer solution of 7.0 (1/15 mol PBS) as one of the tested waters. On the other hand, JSPW and SSW contain about 3.5% NaCl plus small amounts of many other minerals and elements (15). As most bacteria require about 0.9% NaCl for growth, many bacteria appeared to be unable to cope with environments of 3.5% NaCl. We used a 3.5%NaCIPBS to assess the effect of the salt in seawater on the growth of the bacteria. A total of 5 kinds of water (JSPW, SSW, 3.5% NaCIPBS, PBS, and PW) were used in the present study.

Our data have demonstrated that undiluted JSPW had an inhibitory effect on the growth of L. pneumophila SG1 and SG6, E. coli, and S. aureus. The results obtained in L. pneumophila corresponded to the previously reported finding that salt concentrations of 1.5% NaCl or more had a great influence on the survival of L. pneumophila serogroup 3 (21). However, the 10^-2-fold dilute solution of JSPW showed no inhibitory effect on the growth of L. pneumophila SG1, or E. coli. Previous studies have demonstrated that virulent cell
growth of *L. pneumophila* was susceptible to inhibition by relatively low NaCl concentrations and appeared to be completely inhibited by NaCl concentrations higher than 0.4% (22), and the ability of *L. pneumophila* to survive in various concentrations of NaCl and sea water was shown to be enhanced by the addition of small amounts of NaCl (0.1–0.5%), suggesting a protective effect of NaCl (21). The 10^2-fold dilute solution of JSPW among the 3 concentrations we used in this study was the concentration at which *L. pneumophila* SG1 had its optimal survival rate. JSPW contains higher concentrations of nitrite-nitrogen and phosphate-phosphorus, which are inorganic nutrient salts, than SSW (14). This may be one reason the 10^2-fold dilute solution of JSPW showed no inhibitory effect on the growth of *L. pneumophila* SG1 whereas the solution of SSW did.

The possibility that the NaCl concentration is associated with the survival of *E. coli* was suggested based on the fact that the results obtained from *L. pneumophila* SG1 and *E. coli* showed similar tendencies (Tables 1–3). Moreover, an inverse association between the calcium and magnesium concentrations of the water and contamination with *L. pneumophila* has been previous reported (23). JSPW and SSW are richer in calcium and magnesium than PW (15), and they are susceptible to pollution with *L. pneumophila*. However, the salt contained in seawater is bound to inhibit the growth of *L. pneumophila*.

On the other hand, all of the tested water samples showed inhibitory effects on the survival of *S. aureus* at 1 day after incubation or later, although *S. aureus* is relatively resistant to high salt content and also requires many macronutrients and micronutrients for growth. The inhibitory effects may be due to a lack of the nutrients required for growth in the tested water samples, as no nutrients were added to the test tubes.

The pH levels of JSPW and SSW have been examined using a glass electrode method with the following results: JSPW pH, 7.87; SSW pH, 8.25 (15). Despite the different pH values, the results of surviving cells obtained for JSPW were quite similar to those for SSW and 3.5% NaClPBS (pH 7.0). These results suggest that the inhibitory effect of pH on the growth of *L. pneumophila* SG1 and *E. coli* may be smaller than that of NaCl concentration.

In PW and PBS, no inhibitory effects on the growth of *L. pneumophila* SG1 and *E. coli* were found. Therefore, it is possible that these bacteria can survive in environmental water under typical environmental temperature conditions, and so environmental water can be a source of contagion.

Potential limitations of our study warrant discussion. For example, we used bacteria concentrations of approximately 10^3–10^4 cfu/ml for *L. pneumophila* SG6, and 10^4 cfu/ml for *S. aureus*. Since PW showed inhibitory effects on the growth of *L. pneumophila* SG6 and *S. aureus* after 1 day of incubation, these concentrations might be too low to assess the inhibitory effects of PW on the growth of the bacteria. Further study is needed to determine whether PW also shows these inhibitory effects when higher concentrations of bacteria are present. In addition, no nutrients for the growth of the bacteria were added to the test tubes. Under environmental stress, such as the low nutrients and high NaCl concentrations used in the present study, the bacteria may be in a viable but non-culturable (VBNC) state. It is known that, in low-nutrient environments, *L. pneumophila* cells are able to enter the VBNC state (24). Therefore, an examination of the VBNC state of these bacteria under the conditions tested in the present study is essential to prevent the spread of infection. Moreover, bath water becomes contaminated with soaps, shampoos, skin-scales, sweat, and other body secretions that may act as nutrients for bacterial invaders after bathing. Further monitoring tests of the bacterial pollution in bath water is necessary to support our findings. Consequently, our next trial will be the survival test of the bacteria in water samples supplied with nutrients for the bacteria.

We have previously reported the following: hot JSPW bathing might have the ability to change human immune cell distribution, whereas SSW bathing showed no such effect (6). Also, among JSPW, SSW, and PW, JSPW was shown to be mildest to the human body (7). In addition, subjects reported a sticky feeling after SSW bathing, whereas no sticky feeling was reported after JSPW bathing (6, 7). These findings suggest that JSPW is better bath water than SSW.

In conclusion, the current study indicates that the growth of *L. pneumophila* SG1 and SG6, *E. coli*, and *S. aureus* were inhibited in undiluted JSPW for 7 days after incubation at 37°C, just as they are in SSW and 3.5% NaClPBS. In the control PW, however, the surviving cells of *L. pneumophila* SG6 and *S. aureus* had also decreased more than 2 log units after 1 day of incubation. These findings suggest that undiluted JSPW has inhibitory effects on the growth of *L. pneumophila* SG1 and *E. coli*, and that the inhibitory effects may be due to the high concentration of salt in JSPW.

**Acknowledgements**

This study was supported by a Grant-in-Aid from Niigata Prefecture as a part of the Japan Sea Proper Water Utilization Technology Research and Development Program in 2003.

**References**

5. Neumann L, Sukenik S, Bolotin A, Abu-Shakra M, Amir M,


