# (29) INTRACELLULAR SURVIVABILITY OF *Legionella pneumophila* IN VBNC STATE AGAINST SILVER AND COPPER EXPOSURE

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Eentering into the viable but non-cultivable (VBNC) state in a synthetic drinking water (SDW at 50mL, pH 7.0, and 25°C), the intracellular resuscitation in *Acanthamoeba polyphage* (ATCC30462), and survivability of *L. pneumophila* against exposure to silver ($AgNO_3$) and/or copper ($CuSO_4 \cdot 5H_2O$) reagents were investigated in this study. As the results, *L. pneumophila* completely lost its cultivability after 30 days incubation in SDW, while it maintained its viability (determined by using flow cytometry (FCM) with SYTO 9 and propidium iodide staining) at a nearly constant level (0.1 log reduction) for 190 days. After 4 days co-incubation with *A. polyphage* ($1\times10^5$amoeba/mL), *L. pneumophila* in VBNC state recovered its cultivability and started to resuscitate, and finally grown to $2.55\times10^7$CFU/ml after 7 days. The bacteria that resuscitated and multiplied in amoeba was then exposed to silver (0.1mgAg/L) and/or copper (1.0mgCu/L) reagents. All of planktonic *L. pneumophila* was inactivated completely within 8 hours after injection of silver or copper, but $7.3\times10^3$ and $5.6\times10^1$CFU/ml of *L. pneumophila* residing inside of *A. polyphage* survived against copper and silver exposure even after 7days incubation, respectively. Consequently, the *L. pneumophila* residing inside of *A. polyphage* showed much higher tolerance than *L. pneumophilia* in planktonic state against the same doze of silver and/or copper, and this phenomenon was explained by the amoeba interruption to chemisorption of disinfectant onto bacteria residing inside of amoeba.

***Key Words :*** *Legionella pneumophila, VBNC, Acanthamoeba polyphage, resuscitation, silver and copper*

## 1. INTRODUCTION

Of the pathogenic bacteria in water environments, the gram-negative rod-shaped *Legionella pneumophi -la* is generally detected in natural water and can cause legionnaires' disease (one of pneumonia diseases) via the establishment of a replication organelle in vivo[1–4]. Barker and Brown (1992)[5] and other researchers[6–8] demonstrated that *L. pneumophila* released in a natural or artificial water environment was often exposed to stresses due to limitations and changes in nutrient availability, temperature, salinity, oxygen, and pH. In this case, *L. pneumophila* often temporarily enters into a noncultivable state generally referred to as viable but noncultivable (VBNC), in which they regulate cell metabolism to adapt to environmental stresses. This *L. pneumophila* in VBNC state can resuscitate when environmental and/or nutritional conditions are improved[9), 10)].

Dozens of previous research[11), 12)] demonstrated free-living protozoa or amoebae in natural and artificial environments, such as *Acanthamoeba*, *Vahlkampfia*, and *Hartmannella* spp. serve their organisms to *Legionella* spp. and *Pseudomonas* spp. as host in which bacteria multiply in organelle -studded phagosomes. Particularly, intracellular resuscitation of *L. pneumophila* by the endosym-biozation with *Acanthamoeba polyphage* has been widely studied to understand the survival strategy of *L. pneumophila* in starving conditions[6]. Furthermore, Donlan and Costerton[13] demonstrated that bacteria within amoeba cells had much higher resistance against bactericidal reagents than bacteria in planktonic state.

For the inactivation of pathogens, silver and/or copper reagents are recently applied to water disinfection in the laboratories to overcome the drawbacks of the chlorination[14]. Silver compounds such as silver nitrate, silver sulfadiazine, silver acetate, and silver protein have antimicrobial properties and have long been used as microbiocidal agents[15),16)]. Mcdonnell and Russell (1999)[15)] summarized that the mechanism of biocidal action of the silver ion was related to the interaction with thiol (sulfydryl, -SH) groups in enzymes and proteins. Other researchers verified the effects of silver as the release of $K^+$ ions from microorganisms, hydrogen bond breaks, depression of nutrient uptake, inhibition of cell division, interference of proton transfer, and bonding to DNA, which resulted in increased stability of the double helix[13-14), 17)].

The purpose of this study was to investigate the properties of *L. pneumophila* entring into the VBNC state, and intracellular resuscitation in *A. polyphage*. Survivabilities of *L. pneumophila* in the planktonic state and in endosymbiosis state were compared by exposing *L. pneumophila* to silver and copper reagents. Furthermore, the number of bacteria entrapped in amoebae was measured, and was compared with the number of planktonic bacteria.

## 2. MATERIALS AND METHODS

### (1) Model strains and preparations

*Legionella pneumophila* (ATCC 33152) was used as target pathogenic bacteria, and *Acanthamoeba polyphage* (ATCC 30461) was selected as a host cell for intracellular resuscitation of *L. pneumophila* throughout this study. The strain of *L. pneumophila* was cultured in buffered charcoal yeast extract α (BCYE α) medium[11)] (11.5g Yeast extract, 1.5g activated charcoal, 6.0g N-(2-Acetamido) -2-amino -ethanesulfonic acid ($C_4H_{10}N_2O_4S$, ACES) buffer, 1.0g α-ketoglutarate, and 5.0ml *Legionella* agar enrichment (0.2g L-Cysteine HCl, 0.125g ferric pyrophosphate) per litter of distilled water) for 4 days at 35°C. *A. polyphage* was incubated in Peptone yeast-extract glucose (PYG) medium[8)] for 10 days at 25°C. PYG medium was prepared in laboratory before experiments following to the protocol described in American Type Culture Collection (ATCC712 medium). PYG medium was composed with 20.0g Proteose Peptone (BD 211684), 1.0g Yeast extract, 10.0mL of 0.4M $MgSO_4 \cdot 7H_2O$, 8.0mL of 0.05M $CaCl_2$, 34.0mL of 0.1M Sodium citrate·$2H_2O$, 10.0mL of 0.005M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 10mL of 0.25M $Na_2HPO_4 \cdot 7H_2O$, 10.0mL of 0.25M $KH_2PO_4$ and 950mL of distilled water. Then, pH of prepared medium was adjusted to 6.5 with 1N HCl and NaOH, and 50mL of 2M Glucose (filter sterilized) was finally added to medium. Bacterial samples for inoculation were collected in the exponential growth phase by successive monitoring with flow cytometry (FCM) and adjusted to a density of $1 \times 10^7$cells/mL. Samples of *A. polyphage* was also collected at the exponential growth phase by monitoring with the hemocytometer counting method using plankton counter (MPC-2000, Matsunami, Japan).

### (2) Experimental events

A synthetic drinking water[18)] (SDW, Table 1) was used as inoculation medium throughout this study to prevent unknown disturbances on experiments, and three different experimental stages were successively organized to observe the physiological properties of *L. pneumophila*.

First, to investigate the entry into the VBNC state, pure-cultured *L. pneumophila* in exponential phase was inoculated into 50mL of SDW and incubated for 190 days at 25°C without any injection of nutrients or minerals. Before inoculation into SDW, bacteria in BYEC medium was centrifuged (Kubota 5200, Kubota Co. Japan) at 3000rpm for 15min, and the supernatant was discarded to reduce the residual effect of culture media. Then the sample was resuspended in SDW by vortexing, and this washing process was repeated three times. Finally, the bacteria was inoculated to glass vessel involving SDW (50mL) at a density of $1.0 \times 10^5$ cells/mL.

The second condition was the co-incubation of *A. polyphage* with *L. pneumophila* that were incubated in SDW for 190 days. Prior to inoculation of *A. polyphage* into the sample, *A. polyphage* grown in

**Table 1** Compositions of synthetic drinking water

| Compositions | Amount (μg/L) |
|---|---|
| Glucose [a] | 1.00 |
| $KNO_3$ | 995.07 |
| $KH_2PO_4$ | 129.92 |
| $Na_2SO_4$ | 44.29 |
| $CaCl_2 \cdot 2H_2O$ | 18.34 |
| $MgCl_2 \cdot 6H_2O$ | 41.81 |
| $FeCl_3 \cdot 6H_2O$ | 9.68 |
| KCl | 19.07 |
| $CoCl_2 \cdot 6H_2O$ | 0.40 |
| $CuCl_2 \cdot 2H_2O$ | 0.54 |
| $MnSO_4 \cdot 5H_2O$ | 21.94 |
| $ZnCl_2$ | 0.21 |
| $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ | 0.13 |
| pH [b] | 7.00 |

[a] Unit : mgTOC/mL
[b] pH was adjusted by KOH or KCL

PYG medium were washed three times with PY medium (PYG medium without glucose) and SDW to eliminate the remaining nutrients (centrifuging at 1000 rpm for 30min). To reduce the amoebal shock from environmental changes, washing was progressed subsequently with PY and SDW. Then, *A. polyphage* was inoculated into sample at a density of $1.0\times10^5$amoeba/mL and co-incubated for 10days at 25°C in order to observe the resuscitation of *L. pneumophila*.

The subsequent condition was the exposure of *L. pneumophila* that were co-incubated with amoeba cells to silver and copper reagents, successively. Silver originated from silver nitrate, copper originated from copper sulfate (5-hydrate), and a combination of silver and copper reagents were injected into samples and finally adjusted to the concentration of 0.1mgAg/L, 1.0mgCu/L, and (0.1mgAg+1.0mgCu)/L, respectively. Samples containing disinfectants were then incubated under the same conditions with above two experimental conditions for 1 week to observe the survival profiles of the *L. pneumophila*. The concentrations of copper and silver reagents were determined with inductively coupled plasma mass spectrometry (ICP/ MS, HP 4500 series, USA) after pretreatment with nitric acid (3%).

**(3) Measurement of the number of bacteria in planktonic and endosymbiosis states**

The numbers of *L. pneumophila* in a planktonic state and in an endosymbiosis state were differentiated by the application of a filtration and sonication[9), 21)].

The populations of *A. polyphage* ($10^4$~$10^6$ amoeba /mL) were completely elimiated by filtration with 5μm-pore-size membrane filter (Schleicher & Schuell Co. Germany), and no amoebal cell was observed by hemocytometer method after filtration. On the other hands, the number of *L. pneumophila* was not remarkably affected by filtration at the same filtering condition. 8.27 log CFU/mL of *L. pneumophila* was decreased to 8.26 log CFU/mL after filtration, and almost of *L. pneumophila* (99.50%) passed the 5μm-pore-size membrane filter. This meant that *L. pneumophila* in *A. polyphage* cell was completely eliminated by filtration (5μm), therefore, the counted number of colony after filtration (5μm) was regarded as the number of *L. pneumophila* in planktonic state in this study.

The number of *L. pneumophila* residing inside of *A. polyphage* (endosymbiosis state) was calculated by introduction of a sonication with homogenizer (Cup-type, Ultras homogenizer VP-60S, Taitec, Japan) setting with a power of 7W for 60 sec at room temperature. At the range of $10^2$~$10^6$ amoeba/mL, averagely 93.97% of *A. polyphage* was destroyed by sonication with setting conditions. On the other hands, the number of *L. pneumophila* was decreased from 8.3 to 8.2 log CFU/mL, and was not significantly affected by sonication at the same conditions(average 96.17% of *L. pneumophila* was shown in alive after sonication). The number of bacteria in the endosymbiosis state was calculated by subtracting the number of colonies forming on the BCYE agar plate after 5μm filtration from the number of colonies after sonication.

**(4) Detection of the cultivability and viability**

The cultivability of *L. pneumophila* was determined periodically by counting the average number of colonies formed on the surface of BCYE agar plates.

Viability is normally classified depending on the integrity of the cell membrane[1), 19)]. In this study the viability of *L. pneumophila* was determined by using the permeability of two molecular probes, SYTO 9 (permeable) and propidium iodide (PI, impermeable) (LIVE/DEAD *Bac*light Bacterial Viability Kits, L-7012, Molecular Probes, Inc., USA) into cell membrane. A flow cytometer (FCM, EPICS ALTRA flow cytometer, Beckman Coulter Inc., USA) was used to detect the viability after double staining of the nucleic acids of *L. pneumophila* with SYTO 9 and PI. The number of *A. polyphage* was directly counted by microscopic observation with hemocytometer counting method.

**(5) FCM with fluorescently stained cells**

For the fluorescent staining of nucleic acids (DNA/RNA) of *L. pneumophila* cell, SYTO 9 and PI were simultaneously added to the samples at a concentration of 1.5μg/mL of sample. Samples were then incubated at room temperature for 15minutes in the dark. Stained samples were analyzed by using FCM after addition of 100μl Flow count™(assayed concentration was 1052particles/μL, Beckman Coulter, USA) solution to count the bacterial number in viable status. The FCM was equipped with a 488nm argon laser. The emitted fluorescence was split into three different photomultiplier tube (PMT) channels; PMT1 for the flow counter, PMT2 with the wavelength range of 510 to 540nm for SYTO 9, and PMT4 (615 to 640nm) for PI with red fluorescence.

## 3. RESULTS AND DISCISSION

### (1) Entry of *L. pneumophila* into the VBNC state

The variations of cultivability and viability corresponding to the time period were shown in Fig. 1. The initial number of *L. pneumophila* in cultivable condition was measured as $4.4\times10^4$CFU/mL after inoculation into SDW (50mL, pH 7.0). However, *L. pneumophila* rapidly lost its cultivability to $2.0\times10^1$CFU/mL after 14 days incubation, and formed no colonies on BCYE agar plate culture medium after 30 days. In comparison with the cultivable properties, the viability of *L. pneumophila* determined by FCM analysis with double stain of nucleic acids by SYTO 9 and PI was observed constantly with no remarkable variations. The number of *L. pneumophila* in viable condition varied only from $4.7\times10^4$Cells/mL to $4.2\times10^4$Cells/mL (0.1 log reduction, 11%) during the 190-day incubation. Previous studies[9),10)] demonstrated that the lack of minerals could result in the simultaneous loss of cultivability and viability of *L. pneumophila* under extreme starvation conditions such as in distilled water or seawater, but significant reduction of viability was not observed during experimental period. It is considered that the maintenance of viability in this study seems to be based on the presence of trace minerals in SDW. States et al.(1985)[12)] demonstrated that certain minerals such as iron, zinc, and potassium were important factors in the survival of *L. pneumophila* in tap water. In other words, *L. pneumophila* could maintain its viability for a long time by reducing metabolic activities (cultivability) as long as those minerals were present.

As shown in Fig. 1, *Legionella pneumophila* completely entered into the VBNC state after 30 days incubation in SDW. This result was comparable with previous studies. Steinert and coworkers (1997)[6)] demonstrated that *L. pneumophila* JR 32 entered into the VBNC state in sterilized tap water after 125 days. Another research[9)] investigated that *L. pneumophila* serogroup 1 suzuki entered into the VBNC state after 60 days in hot spring water at 25 °C, and it was varied corresponding to the change of pH and temperature.

### (2) Endosymbiozation with amoeba and intracellular growth of *L. pneumophila*

The properties of the intracellular resuscitation of *L. pneumophila* in VBNC state according to the co-incubation with *A. polyphage* was shown in Fig. 2. The number of *L. pneumophila* both in planktonic and endosymbiosis states did not rapidly change for 2 days. However, *L. pneumophila* started to penetrate into amoeba (endosymbiozation) cell and multiplied exponentially after 2days incubation with *A. polyphage*, and finally grown to $1.7\times10^7$ CFU/mL within 10 days. The resuscitation and multiplication of *L. pneumophila* in *A. polyphage* was followed by increase of *L. pneumophila* in planktonic state to $2.6\times10^7$ CFU/mL. During this period, the population of *A. polyphage* reduced from $1.3\times10^5$ to $5.1\times10^2$ amoeba/mL (2.4 log reduction), respectively (Fig. 3).

After the 10-day co-incubation of *L. pneumophila* with *A. polyphage*, the number of *L. pneumophila* in

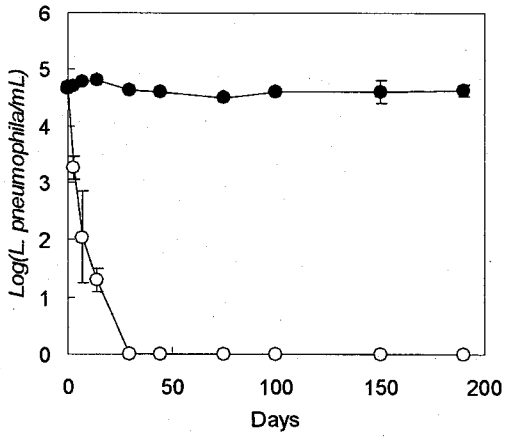


**Fig. 1** Observed bacterial cultivability and viability of *L. pneumophila*. Symbol ● indicated the number of viable *L. pneumophila* detected by flow cytometric analysis (FCM) with double fluorescent staining of nucleic acid, and ○ indicated the number of cultivable *L. pneumophila* detected by plate culture colony counting method.

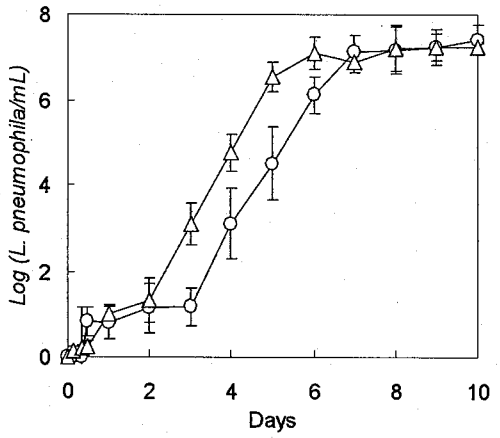


**Fig. 2** Intracellular resuscitation of *L. pneumophila*. Symbol △ indicated the number of *L. pneumophila* in endosymbiozation state, and ○ indicated the number of *L. pneumophila* in planktonic state detected by plate culture colony counting method (following after sonication in the case of Symbol○).

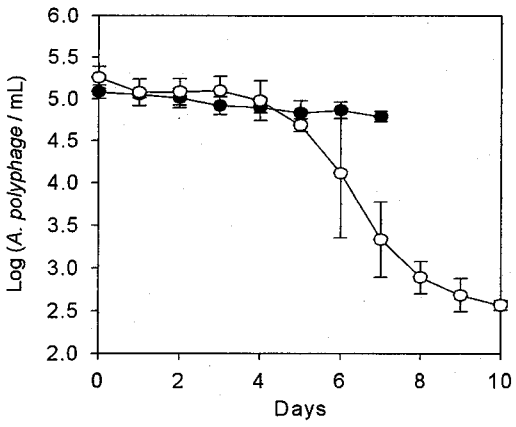


**Fig. 3** Variation of the population of *A. polyphage*. Symbol ○ indicated the population of amoeba when co-incubated with *L. pneumophila*, and ● indicated the population of amoeba when only *A. polyphage* was incubated in SDW without any injection of bacteria (control test).

endosymbiosis state increased to $1.7\times10^7$ CFU/mL, and the number *A. polyphage* was $5.1\times10^2$ amoeba/mL at that time. Based on the division of the number of bacteria in endosymbiosis state by the number of amoeba, the number of *L. pneumophila* cells that was contained inside of an *A. polyphage* was estimated. As a result, $3.4\times10^4$ cells of *L. pneumophila* resided in each cell of *A. polyphage*. This calculated value was comparable with previous studies. Barker et al. (1992)[5] demonstrated that the number of bacteria in the amoeba depends on the size of the amoebae vesicle or cyst. Berk et al. (1998)[7] calculated that each vesicle of *A. polyphage* could contain between 20 and 200 *L. pneumophila* cells, and another researcher[13] calculated from 365 to 1,483 bacteria in a 5 μm diameter vesicle by using the similar methods with Berk et al.(1997)[7].

The number of *L. pneumophila* contained in an amoeba cell was calculated at 10-1,000 times bigger than previous studies, and this difference seemed to be originated from the difference of calculating method. This study calculated the number of bacteria in a amoeba based on the number of each microorganism, but mentioned previous studies used the mathematical equation[7] based on the size of amoeba to calculate the number of bacteria in amoeba.

### (3) Intracellular survivability against silver and copper exposure

To compare the survivability of *L. pneumophila* in two states (planktonic state and within amoeba state) The samples of *L. pneumophila* were exposed to silver (0.1mgAg/L as $AgNO_3$), copper (1.0mgCu/L as $CuSO_4$), and their combination (0.1mgAg +1.0mgCu/L). Fig. 4 showed the survival properties of *L. pneumophila* in planktonic state and endosymbiosis state corresponding to the time period. After exposure to disinfectant, *L. pneumophila* in planktonic state were completely inactivated (7.2 log reduction was detection limit) and did not form any colonies after less than 8 hours exposure to silver or copper. However, the number of *L. pneumophila* residing inside of *A. polyphage* did not reduce as much as the number of bacteria in the planktonic state even

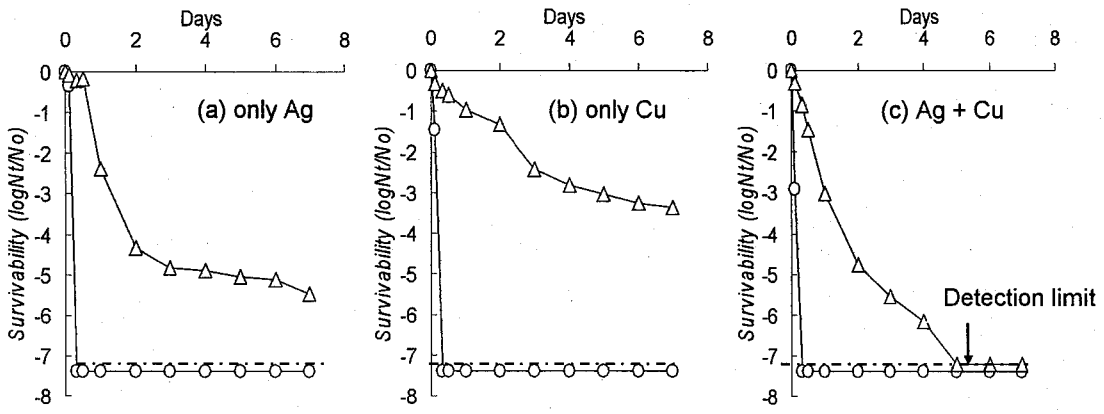


**Fig. 4** Intracellular survival profiles of *L. pneumophila* in the planktonic state and within *A. polyphage* state against copper or silver exposure. Symbol △ indicated the survivability of *L. pneumophila* in amoeba state and symbol ○ indicated in planktonic state. Left graph indicated the date obtained when only silver was inoculated, middle graph showed when only copper was injected, and right one illustrated the results when both silver and copper were combined.

after 7 days exposure to silver and copper. Actually, *L. pneumophila* inside of amoeba survived at a level of $7.3\times10^3$ against copper, and $5.6\times10^1$ CFU/mL against silver exposure after 7 days exposure, respectively. During the same period, the number of *L. pneumophila* without any injection of silver and/or copper reagents (Control test) was varied from 7.2 to 6.4 log CFU/mL (planktonic state), and from 7.2 to 6.8 log CFU/mL (endosymbiosis state), respectively.

Benarde and coworkers (1967)[20] differentiated the biocidal mechanism of disinfectant as 3 steps, (i) mass transfer in the liquid to target bacteria liquid interface, (ii) chemisorption of the disinfectant at selective active centers on the cell surface, and (iii) surface and intrasurface diffusion of the activated chemisorbed complex with attendant chemical attack on cellular elements. It is considerable that the endosymbiozation of *L. pneumophila* with *A. polyphage* might allow to delay the time for chemisorption of silver and copper at bacterial surface and decreasing the amount of disinfectant that attack on cellular elements of *L. pneumophila*. Mcdonnell and Russell(1999)[15] also demonstrated that bacteria within the amoeba were resistant to chemical disinfectants might be because amoeba cysts were more impermeable to disinfectant molecules than vegetative cells. Previous researchers have reported similar experimental results related to the intracellular resistance against antimicrobial reagents. Barker et al.(1992)[5] reported that 50-100% of *L. pneumophila* cells grown within *A. polyphage* could survive, but 99.99% of bacteria in the planktonic state inactivated after 4 hours exposure to polyhexamethylene biguanide (PHMB). Donlan and Costerton(2002)[13] summarized that 99% inactivation of planktonic *L. pneumophila* was accomplished by exposure to 0.5mgCl/L for 1minute, but 10mgCl/L for 2 hours (pH 7.0, 25°C) was required to inactivate 99% of *L. pneumophila* entrapped in *Acanthamoeba castellani*.

Although the concentration of silver (0.1mgAg/L) injected in SDW was 10 times lower than the concentration of copper (1.0mgCu/L) reagent, the biocidal capacity of the silver reagent for inactivation of model bacteria within the amoeba observed much higher than copper. For example, 5 log reduction of the number of *L. pneumophila* was detected at the silver exposure after 7 days incubation (Fig. 4 (a)), while just 3 log reduction was measured at copper case (Fig. 4 (b)), respectively. Additionally, survivability of *L. pneumophila* was remarkably reduced when the two biocidal reagents were simultaneously combined. Though certain number of *L. pneumophila* was survived in *A. polyphage* even after 7 days exposure to silver or copper (Fig. 4 (a) and (b)), *L. pneumophila* in endosymbiosis state was completely inactivated within 5 days exposure to silver and copper combination (Fig. 4 (c)). States et al. (1985)[12] also demonstrated the synergistic effect of the combination of silver with copper reagents.

## 4. Conclusion

In this research, three kinds of different experimental condition was successively organized to investigated the survival strategy of *L. pneumophila* against the starving environment, and intracellular resuscitation of *L. pneumophila* in *A. polyphage*. Then, intracellular resistance of *L. pneumophila* against exposure to silver and copper was compared with that of bacteria in planktonic state. *L. pneumophila* lost its cultivability completely after 30 days incubation in SDW and entered into VBNC state to maintain their survivability against lack out nutrients in water environment. The cultivability of *L. pneumophila* in VBNC state was recovered after co-incubation with *A. polyphage*. The cultivability of *L. pneumophila* increased from no colony formation to $1.7\times10^7$CFU/ml, respectively. Furthermore, *L. pneumophila* in *A. polyphage* showed much higher resistance than bacteria in planktonic state against the same doze of silver and copper. The number of *L. pneumophila* in planktonic state showed 7.4 log reductions (under the detection limit) against copper and silver exposure within 30min after injection. However, bacteria in endosymbiosis state survived to $7.3\times10^3$, and $5.6\times10^1$CFU/ml even after 7days, respectively.

Consequently, the *L. pneumophila* residing inside of *A. polyphage* showed much higher tolerance than that in planktonic state against the same doze of silver and/or copper. This phenomenon was explained by the amoeba interruption to chemisorption of disinfectant into bacteria residing inside of amoeba.

**ACKNOWLEDGMENT:** This study was funded by Grant-in-Aid for Young Scientists (A) 17686045, Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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**(Received 26 May, 2006)**