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Note

Effective proliferation of low level *Legionella pneumophila* serogroup 1 cells using coculture procedure with *Acanthamoeba castellanii*

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Abstract

The multiplications of low level *Legionella pneumophila* serogroup 1 cells by the coculture procedure with *Acanthamoeba castellanii* were tested in five strains. The cells in all strains proliferated effectively for isolating. This procedure might be a useful means of improving the successful isolation from environmental and clinical specimens of low level *Legionella* cells, and pursuing the source of infection.

Keywords: *Acanthamoeba castellanii*; Coculture procedure; Intracellular proliferation; *Legionella pneumophila*

Legionella infection occurs mainly by inhalation of aerosols generated from water sources containing *Legionella* such as water distribution systems, communal baths, and cooling towers (Winn, 1999). *Legionella pneumophila* has been detected worldwide as a relatively common pulmonary pathogen, and most infection cases are due to serogroup 1 (Helbig et al., 2002; Yu et al., 2002; National Institute of Infectious Diseases of Japan, 2003; Doleans et al., 2004). This microbe is a ubiquitous inhabitant of the aquatic environment, where it probably survives and multiplies within protozoa such as amoebae in nature (Moffat and Tompkins, 1992; Cirillo et al., 1994). Among the several *Legionella* detection methods, culturing remains time consuming and labor intensive. Recently, DNA detection techniques have shown promise for the rapid diagnosis of *Legionella* infection, and provide results within a short time frame (Rantakokko-Jalava and Jalava, 2001; Declerck et al., 2003). Despite these recent advances in molecular technology, culturing is still considered the gold standard for *Legionella* detection from the environment (Bartie et al., 2003). In clinical specimens, this assay remains also the method of choice (Murdoch, 2003).

As *Legionella* infections occur, culturing is indispensable to pursue the source of infection and establish a transmission pathway for prevention measures, because serotyping and genotyping isolates has become a helpful tool (Miyamoto et al., 1997; Jonas et al., 2000; Nakamura et al., 2003). Cocultivation of samples with amoebae has been demonstrated to be a convenient method for the recovery of *Legionella* spp. from clinical and environmental specimens (Rowbotham, 1983; Wadowsky et al., 1988; Fallon and Rowbotham, 1990;

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Wadowsky et al., 1991; Sanden et al., 1992; La Scola et al., 2001). However, detailed proliferating data in extremely low level *Legionella* cells by coculture with amoebae have been lacking.

Five strains (Nos. 1–5) of *L. pneumophila* serogroup 1 were used. These were separately isolated from hot spring spa water in Hiroshima prefecture, Japan during 2002–2004. Cultures of these isolates grown from single colonies were stored in vials containing porous beads (MicroBank; Pro-Lab Diagnostics, Canada) at −70 °C. Bacteria were grown on buffered charcoal yeast extract agar supplemented with L-cysteine (BCYEα agar pH 6.9: BD; Sparks, MD, U.S.A.) at 37 °C for 4 days. The *L. pneumophila* cells were harvested with sterile distilled water and adjusted to a concentration of 10^8 cells ml^{-1} as the origin of the experimental sample by measuring the optical density at 550 nm with a Densimat spectrophotometer (bioMérieux; Roma, Italy) until it reached 0.125.

Acanthamoeba castellanii ATCC 30234 was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). *A. castellanii* was cultured in 175-cm^2 tissue culture flasks (Nunc; Roskilde, Denmark) with 50 ml of ATCC Medium 712 [2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4; pH 6.5] at 25 °C. After overnight incubation, cultures of *A. castellanii* were washed twice with *Acanthamoeba* buffer (AC buffer; ATCC Medium 712 without proteose peptone, yeast extract, and glucose), harvested by rapping the flask sharply to bring them into suspension, and transferred to a 50-ml centrifuge tube. The cells were centrifuged at 600 ×*g* for 5 min, and the supernatant was removed. The amoeba cells were adjusted to a titer of 10^6 cells ml^{-1} with AC buffer. Then, 5 ml of cell suspension was pipetted into each 15-ml polypropylene centrifuge tube (Iwaki; Tokyo, Japan). After centrifugation at 600 ×*g* for 5 min, the supernatant in each tube was replaced with a 5-ml aliquot from the adjusted sample of *L. pneumophila* which was diluted with AC buffer. At the same time, a 0.1 ml aliquot from the same sample was inoculated on each of 10 plates of BCYEα agar and incubated at 37 °C for 7 days to confirm the initial inoculum numbers of *L. pneumophila* cells. The tubes were incubated with an inclination at 30 °C for 2 h. After 24, 48, and 72 h at 37 °C incubations, the amoeba cells were harvested from the tube by vigorous vortexing. A 0.1 ml aliquot from the cell suspension and the 10-fold serial dilute solutions were inoculated on BCYEα agar plates in duplicate and incubated at 37 °C for 7 days.

Fig. 1 shows the multiplication of five strains by coculture procedure with *A. castellanii*. As the starting numbers of *Legionella* cells tested were from 10 to less than 100 CFU ml^{-1}, the proliferations of strains showed averages of 3.21, 5.88, and 7.56 log CFU ml^{-1} after 24, 48, and 72 h, respectively [Fig. 1(A)]. Inoculations of cells at densities which were from one to less than 10 CFU ml^{-1} resulted in slight decrease of multiplication compared with the larger size of the initial inoculation [Fig. 1(B)]. In practice, none of the microbes at this level can be effectively isolated from 0.1-ml volumes of samples with the conventional method because of there

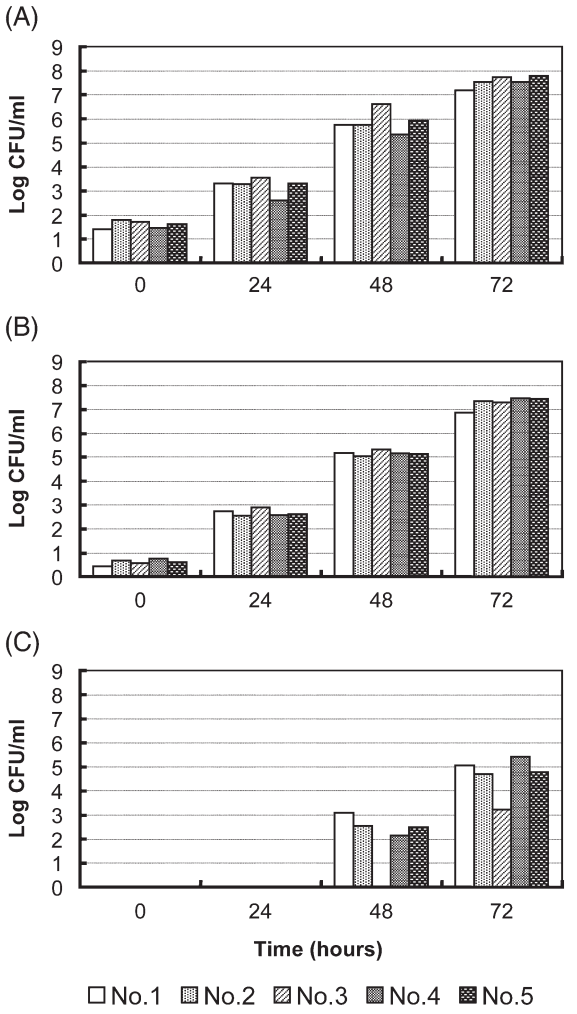

Fig. 1. Multiplications of *L. pneumophila* serogroup 1 strains (Nos. 1–5) by coculture procedure with *A. castellanii* are shown. (A) The starting numbers of cells were from 10 to less than 100 CFU per ml. The coculture assay was performed in duplicate. (B) The starting cells were from one to less than 10 CFU per ml. The coculture assay was performed five times. (C) The starting cells were less than 1 CFU per ml. The coculture assay was performed in duplicate.