

Impact of Free-Living Amoebae on Presence of *Parachlamydia acanthamoebae* in the Hospital Environment and Its Survival *In Vitro* without Requirement for Amoebae[∇]

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Parachlamydia acanthamoebae is an obligately intracellular bacterium that infects free-living amoebae and is a potential human pathogen in hospital-acquired pneumonia. We examined whether the presence of *P. acanthamoebae* is related to the presence of *Acanthamoeba* in an actual hospital environment and assessed the *in vitro* survival of *P. acanthamoebae*. Ninety smear samples were collected between November 2007 and March 2008 (trial 1, $n = 52$) and between October 2008 and February 2009 (trial 2, $n = 38$) from the floor (dry conditions, $n = 56$) and sink outlets (moist conditions, $n = 34$) of a hospital. The prevalences of *P. acanthamoebae* DNA in the first and second trials were 64.3% and 76%, respectively. The prevalences of *Acanthamoeba* DNA in the first and second trials were 48% and 63.1%, respectively. A statistical correlation between the prevalence of *P. acanthamoebae* and that of *Acanthamoeba* was found (trial 1, $P = 0.011$; trial 2, $P = 0.022$), and that correlation increased when samples from just the dry area (floor smear samples, $P = 0.002$) were analyzed but decreased when samples from a moist area were analyzed ($P = 0.273$). The *in vitro* experiment showed that, without *Acanthamoeba*, *P. acanthamoebae* could not survive in dry conditions for 3 days at 30°C or 15 days at 15°C. Thus, both organisms were coincidentally found in an actual hospital environment, with the presence of *Acanthamoeba* having a significant effect on the long-term survival of *P. acanthamoebae*, suggesting that this potential human pathogen could spread through a hospital environment via *Acanthamoeba*.

Chlamydiae, which are obligate intracellular bacterial pathogens, have been reclassified as the order *Chlamydiales*, which includes four families: *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae*, and *Simkaniaceae* (5). The family *Chlamydiaceae* is well known to have a broad range of distribution in animals and humans and to be the causative agents of human diseases (4, 20, 24, 31, 36). This family includes two major human pathogens: *Chlamydomphila pneumoniae*, a causal agent of common respiratory infection and also suspected of being involved in some chronic diseases, such as asthma and atherosclerosis (6), and *Chlamydia trachomatis*, responsible for sexually transmitted disease and preventable blindness (33).

Parachlamydiaceae, *Waddliaceae*, and *Simkaniaceae* have recently been recognized as chlamydiae that exhibit a wide range of distribution in the natural environment, such as in rivers and

in soil (9). All these species can grow and survive dependently within the free-living amoeba *Acanthamoeba*, the most abundant genus of amoeba (1, 9, 11, 12). *Parachlamydia acanthamoebae* and *Simkania negevensis* have been associated with lower respiratory tract infections (3, 27), and *Waddlia chondrophila*, which was originally isolated from an aborted bovine fetus, is considered a potential abortogenic agent (9). There is accumulating evidence supporting the pathogenic role of *P. acanthamoebae* in humans (3, 9, 13). Several studies have reported that parachlamydial DNA was detected by PCR in mononuclear cells of sputa and in bronchoalveolar lavage samples from a patient with bronchitis (10). Other studies have suggested that *P. acanthamoebae* may cause inhalation pneumonia and be responsible for hospital-acquired pneumonia in HIV-infected patients and organ transplant recipients receiving immunosuppressive therapy (7, 8, 14). Thus, *P. acanthamoebae*, presumably spreading through amoebae, is emerging as a potential etiological agent of hospital-acquired pneumonia. However, the overlap between the distribution of *P. acanthamoebae* and that of *Acanthamoeba* in hospitals and the survival of the bacteria in harsh conditions without *Acanthamoeba* remain unknown. We examined whether the prevalence of *P. acan-*

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thamoebae correlates with that of *Acanthamoeba* in a hospital in Sapporo, Japan. We also examined the *in vitro* survival of *P. acanthamoebae* and its requirement for *Acanthamoeba*.

P. acanthamoebae Bn₉ (ATCC VR-1476) was purchased from the American Type Culture Collection. The bacteria were propagated in an amoeba cell culture system according to methods described previously (17). The numbers of infective progeny were determined according to the procedure described below. Free-living amoebae, *Acanthamoeba castellanii* C3 (ATCC 50739), were purchased from the American Type Culture Collection. Amoebae were maintained in PYG broth (0.75% [wt/vol] peptone, 0.75% [wt/vol] yeast extract, and 1.5% [wt/vol] glucose) at 30°C (35). The numbers of infective progeny of *P. acanthamoebae* were determined by a procedure known as the amoeba infectious unit (AIU) assay using coculture with amoebae as described previously (28).

Ninety smear samples were obtained from the floor ($n = 56$) and sink outlets ($n = 34$) of a hospital (Hokkaido University Hospital) containing approximately 900 beds (number of outpatients, approximately 3,000 per day; number of hospital patients, approximately 1,000 per day) in a 12-story building located in the central area of Sapporo, Japan, from November 2007 to March 2008 (trial 1, $n = 52$) and from October 2008 to February 2009 (trial 2, $n = 38$). The samples were collected by wiping an approximately 1-m² area on the floor or sink outlet with sterilized gauze (approximately 25 cm²) moistened with Page's amoeba saline (PAS) (30). Each piece of gauze was then vortexed for 60 s in 20 ml sterilized PAS containing 0.05% (vol/vol) Tween 80, and the suspension was then centrifuged at $2,100 \times g$ for 20 min. Pellets were resuspended in 200 μ l PAS and then used for *P. acanthamoebae* culture and DNA extraction.

One-half of the resuspended smear pellet (100 μ l) was used for *P. acanthamoebae* culture. Culture detection was performed by a method based on amoeba lysis described previously (18). In brief, serially diluted sample solution was added to 100 μ l of amoeba suspension containing 1×10^5 *A. castellanii* C3 cells in 1 well of a 96-well microplate and incubated for up to 10 days at 30°C in a normal atmosphere. The microplate was read daily to determine the highest dilution of bacteria that led to amoeba lysis.

The DNA extraction was performed using the UltraClean soil DNA extraction kit (MBL, Carlsbad, CA) according to the manufacturer's instructions. The primers used in PCR amplification were as follows: Pac1 (5'-GAG GTG AAG CAA ATC CCA AA-3') and Pac2 (5'-CTC CTT GCG GTT AAG TCA GC-3') for amplification of *P. acanthamoebae* 16S rRNA (191 bp), JDP1 (5'-GGC CCA GAT CGT TTA CCG TGA A-3') and JDP2 (5'-TCT CAC AAG CTG CTA GGG AGT CA-3') for amplification of 18S rRNA from *Acanthamoeba* species (423 to 551 bp) (34), and Bac11 (5'-GAG GAA GGT GGG GAT GAC GT-3') and Bac12 (5'-AGG CCC GGG AAC GTA TTC AC-3') for amplification of bacterial 16S rRNA excluding the order *Chlamydiales* (216 bp) (38). The primers for amplification of *P. acanthamoebae* 16S rRNA were designed based on GenBank cDNA sequences (accession number NR026357) using the program Primer 3 (<http://frodo.wi.mit.edu/primer3/input.htm>). To overcome inhibition of PCR amplification by humic acid, bovine serum albumin (BSA) was added to each reaction mixture according to methods de-

scribed previously (23, 39). The quality of extracted DNA was confirmed by PCR amplification using universal primers that target bacterial 16S rRNA, which is conserved across a broad spectrum of bacteria. All smear samples ($n = 90$) yielded PCR amplicons of the expected size and were then used in specific *P. acanthamoebae* and *Acanthamoeba* PCRs. Search results returned by the BLAST program showed that the primers used for each PCR were specific for *P. acanthamoebae* and *Acanthamoeba* detection. The amount of template DNA used in each PCR was 2 μ l. The total reaction volume was 25 μ l and consisted of 200 μ M each deoxynucleoside triphosphate (dNTP), 10 μ M BSA, $1 \times$ commercial reaction buffer, and 0.625 U *Taq* DNA polymerase (New England Biolabs, Herts, United Kingdom). The thermal cycling profile involved an initial denaturation step at 94°C for 10 min; 35 cycles, each consisting of 30 s of denaturation at 94°C and 30 s of annealing at 60°C for *P. acanthamoebae* 16S rRNA, 60°C for *Acanthamoeba* species 18S rRNA, and 52°C for bacterial 16S rRNA; and a 45-s extension at 72°C. The amplified products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The presence of amplified target genes in randomly selected positive specimens was confirmed by direct oligonucleotide sequencing of the PCR products (Macrogen, Seoul, South Korea). As a quality control for each PCR, diluted DNA extracted from the *P. acanthamoebae* Bn₉ or *A. castellanii* C3 strain was used in each amplification. As a negative control, DNA-free water (Sigma) was also used in amplification. To prevent contamination, the preparation of the PCR mixture was performed in a separate room. Alignment analysis and the construction of a phylogenetic tree for *P. acanthamoebae* amplicons ($n = 4$) with previously reported *Parachlamydiaceae* sequences were performed using Genetyx-Mac software (version 10.1) and the neighbor-joining method in MEGA software (version 4). The detection limits of the PCR for *P. acanthamoebae* 16S rRNA and *Acanthamoeba* 18S rRNA were examined by using DNA extracted from the sterilized gauze moistened with PAS that had been spiked with defined numbers of *P. acanthamoebae* AIU and *Acanthamoeba* cells, respectively. The detection limits of the PCR targeted to *P. acanthamoebae* and *Acanthamoeba* were 10^2 AIU and 10 cells, respectively.

The procedure for monitoring bacterial viability was as follows. A bacterial solution of 100 μ l containing approximately 10^7 to 10^8 AIU prepared with PAS was placed into wells of a 24-well plate with (moist conditions) or without (dry conditions) 900 μ l PAS, and the plates incubated for up to 28 days at 15°C or 30°C in a normal atmosphere. At 0, 3, 7, 15, and 28 days after inoculation, the supernatant in each well was collected and centrifuged at $3,500 \times g$ for 30 min. In the case of the samples for moist conditions, supernatants were directly transferred to a centrifuge tube. For the dry condition samples, the wells were washed with 900 μ l PAS and this was then transferred to a centrifuge tube. The resulting bacterial pellet was resuspended in 100 μ l PAS. The numbers of infective progeny as a marker of bacterial viability in the solution were determined by the AIU assay as described above. The bacterial membrane integrity as a possible indicator for bacterial viability was also confirmed with fluorescence microscopy by using a Live/Dead reduced biohazard viability/cytotoxicity kit (Molec-

TABLE 1. Correlation between prevalence of *P. acanthamoebae* and that of *Acanthamoeba* spp. in the hospital environment

Sampling period or site	Result for <i>Acanthamoeba</i> sp. DNA (n)	No. (%) of samples for <i>P. acanthamoebae</i> DNA testing:		<i>p</i> ^a
		Positive	Negative	
November 2007–March 2008 (1st trial; n = 52)	Positive (25) Negative (27)	18 (64.3) 10 (35.7)	7 (29.2) 17 (70.8)	0.011*
October 2008–February 2009 (2nd trial; n = 38)	Positive (24) Negative (14)	19 (76) 6 (24)	5 (38.4) 8 (61.6)	0.022*
Sink outlet (moist condition; n = 34) ^b	Positive (11) Negative (23)	6 (42.9) 8 (57.1)	5 (25.0) 15 (75.0)	0.273
Floor (dry condition; n = 56) ^b	Positive (39) Negative (17)	32 (82.1) 7 (17.9)	7 (41.2) 10 (58.8)	0.002*

^a Correlation between the frequency of *P. acanthamoebae* and that of *Acanthamoeba* was analyzed by Fisher's exact test. Asterisks indicate statistically significant results.

^b Mixture of 1st and 2nd trials.

ported *P. acanthamoebae* sequences (concordance rate, 96.3%) (Fig. 1B), and the sequences of amplicons formed a cluster on the phylogenetic tree under the family *Parachlamydiaceae* (Fig. 1C). As shown in Table 1, the prevalences of *P. acanthamoebae* DNA in the first and second trials were 64.3% and 76%, respectively. The prevalences of *Acanthamoeba* DNA in the first and second trials were 48% and 63.1%, respectively. A statistical correlation between the prevalence of *P. acanthamoebae* and that of *Acanthamoeba* was found (trial 1, *P* = 0.011; trial 2, *P* = 0.022). No difference in prevalence between the sampling periods was observed, and the correlation became much higher when floor smear samples were separately analyzed (*P* = 0.002). Interestingly, the prevalence of both organisms in the hospital statistically significantly decreased toward the upper floors (Fig. 2). Successful culture of *P. acanthamoebae* was not achieved.

In vitro survival of *P. acanthamoebae* without *Acanthamoeba*.

To evaluate the *in vitro* survival of *P. acanthamoebae* under conditions lacking amoebae, *P. acanthamoebae* was incubated without amoebae in moist and dry conditions. As shown in Fig. 3B, the numbers of *P. acanthamoebae* progeny in moist conditions, regardless of culture temperature, were stably maintained for up to 28 days. The numbers of *P. acanthamoebae* progeny grown in dry conditions rapidly decreased, with the bacterial numbers declining more rapidly at 30°C than at 15°C. We also confirmed by Live/Dead staining analysis that *P. acanthamoebae* membrane integrity as a possible indicator for viability in moist conditions was maintained during this culture period but not in dry conditions (Fig. 3A).

The emergence of *P. acanthamoebae* as a novel pathogenic agent of respiratory tract infections in hospital environments has important implications for preventing and controlling hospital-acquired diseases (3, 8, 9, 10, 14). Several *in vitro* studies have shown that *P. acanthamoebae* grew and multiplied in free-living *Acanthamoeba* amoebae, which are distributed in a broad range of natural environments but also in the water systems of hospitals (1, 12–15). Whether *P. acanthamoebae* could survive in an actual environment within *Acanthamoeba* spp., which are considered a protective reservoir for bacteria from multiple environmental stresses, remains to be seen. We hence attempted to determine the effects on *P. acanthamoebae*

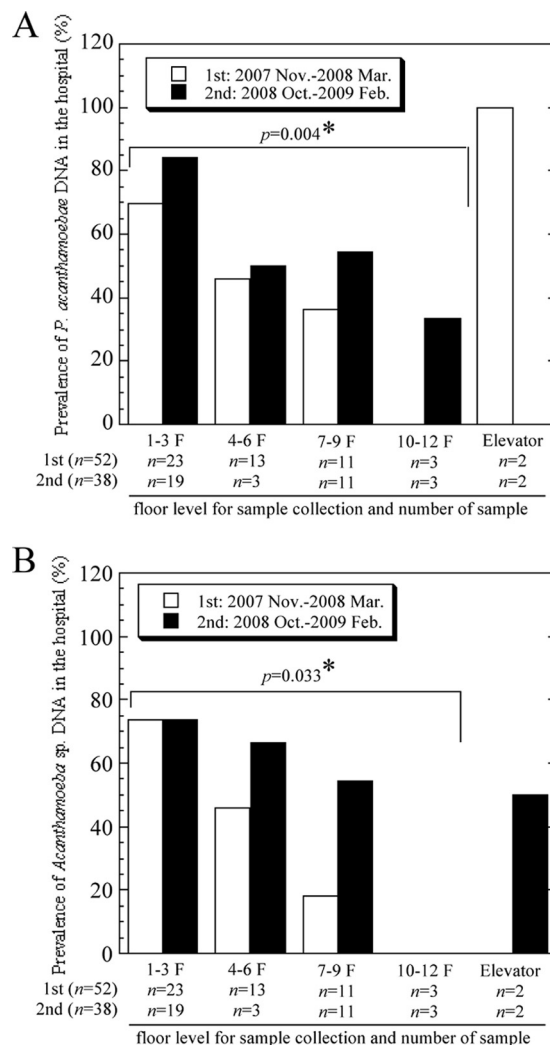


FIG. 2. Influence of floor level on the prevalences of *P. acanthamoebae* (A) and *Acanthamoeba* (B). Asterisks indicate statistical significance.

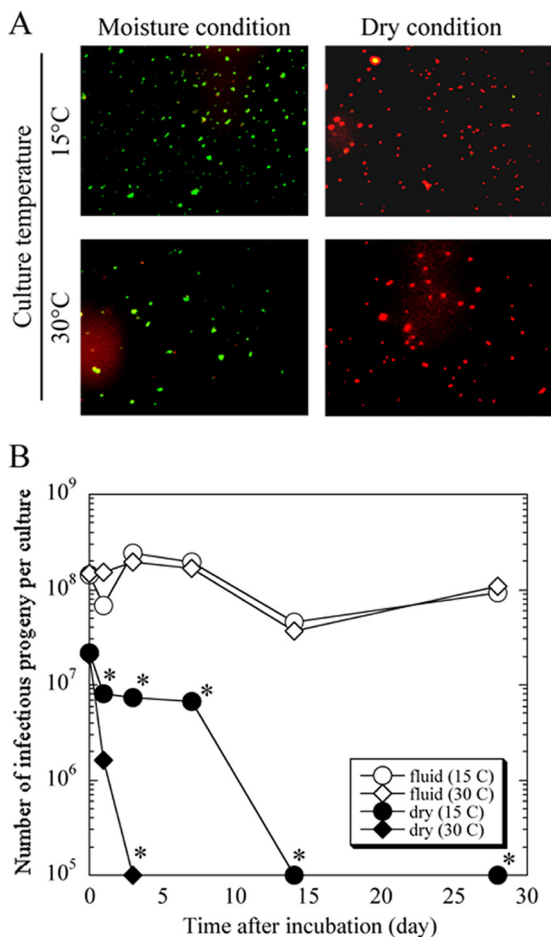


FIG. 3. Representative images of Live/Dead staining in *P. acanthamoebae* (A) and numbers of *P. acanthamoebae* infective progeny in cultures without amoebae (B). (A) The bacteria at 28 days after incubation were stained with a Live/Dead staining kit. Green, membrane integrity stable (possibly viable bacteria); red, dead bacteria. Magnification, $\times 400$. (B) A bacterial solution of 100 μ l containing approximately 10^7 to 10^8 AIU prepared with PAS was inoculated into the wells of a 24-well plate with (moist conditions) or without (dry conditions) 900 μ l of PAS and incubated for up to 28 days at 15°C or 30°C in a normal atmosphere. The numbers of infective progeny as a marker of bacterial viability in the solution were determined using the AIU assay. The data represent the average AIU counts \pm standard deviations. *, $P < 0.05$.

prevalence and survival due to the interaction of *P. acanthamoebae* with *Acanthamoeba* in the hospital environment.

The use of standard culture techniques to detect microorganisms in complex biological samples obtained from hospital surfaces, such as the floor and sink outlets, is limited when the target population is in a minority because most microorganisms in the environment shift to a nonculturable stage. Thus, the molecular technique of PCR, which can be applied to analyze the entire microorganism community, was used for this study. Since it is known that humic acid, which is ubiquitous in natural environments, inhibits PCR amplification (23, 29), BSA was added to each reaction mixture according to methods described previously (23, 29). No amplification of bacterial 16S rRNA or amoeba 18S rRNA was observed from our smear samples without the addition of BSA (data not shown).

Although the prevalences of *P. acanthamoebae* and *Acanthamoeba* in the hospital environment were very high and there was a significant correlation between the prevalence of *P. acanthamoebae* and that of *Acanthamoeba*, how these organisms came to be inside a hospital remains unknown. Interestingly, the prevalences of both organisms statistically changed depending on floor level, with the prevalences on lower floor levels much higher than those on upper levels. It was also confirmed that the prevalences of both organisms in a total of 75 soil samples collected from public parks (75 places) located in an approximately 10-km² area around the hospital, which is located in the central area of Sapporo, were 72% (54/75) and 92% (69/75), respectively, and that a significant correlation between the prevalence of *P. acanthamoebae* and that of *Acanthamoeba* species was found, as in the hospital experiments (data not shown). Taken together, these results imply that *P. acanthamoebae*, which is a potential human pathogen, can enter from outside a hospital and spread throughout the hospital courtesy of an *Acanthamoeba* host. Meanwhile, we could not isolate *P. acanthamoebae* using an amoeba coculture system, but Thomas et al. have reported that *Chlamydia*-related organisms in 200 samples obtained from a water system could be detected using an amoeba coculture method with amoeba strain *A. castellanii* ATCC 30010C (37). Although the exact reason for the contradiction between our results and their findings remains unknown, it is possible that usage of different amoeba strains is associated with these results. In addition, as mentioned above, the preparation of the PCR mixture was performed in a separate room to prevent DNA contamination, so the possibility of a false positive is negligible.

Nevertheless, it is not clear how the bacteria are able to survive the oligotrophic conditions of a hospital environment. The finding that a correlation between the prevalence of *P. acanthamoebae* and that of *Acanthamoeba* was much stronger in dry conditions provided a hint, suggesting a difference in this bacterium's stability depending on habitat. As expected, the *in vitro* experiment indicated that the stability of the bacteria under artificial dry conditions in the absence of amoebae was not preserved compared to that in moist conditions, suggesting that the protection and survival of *P. acanthamoebae* through *Acanthamoeba* in the hospital environment would be limited, particularly in dry conditions. In contrast, *P. acanthamoebae* grown under moist conditions persistently maintained viability for the culture period, suggesting that *P. acanthamoebae* would be relatively stable in moist environments without the requirement for amoebae for an extended period of time.

The mechanism by which *P. acanthamoebae* can survive in moist environments without the requirement for amoebae remains unknown. As stated above, the order *Chlamydiales* includes the pathogenic chlamydiae, such as *Chlamydia pneumoniae* and *Chlamydia trachomatis*, and the environmental chlamydia *P. acanthamoebae*. These similarly exhibit reduced central metabolic and biosynthetic pathways and are auxotrophic for most amino acids and nucleotides (18). A notable exception is the tricarboxylic acid (TCA) cycle, which is incomplete in the pathogenic chlamydiae (18). Admittedly, the detection of *P. acanthamoebae* by PCR is not definitive proof of the actual presence of viable *P. acanthamoebae* organisms. Culturing is the gold standard for detection of *P. acanthamoebae* from samples; therefore, this was also performed using a

method based on amoeba lysis as described previously (15). However, there were no positive cultures from the smear samples collected from the locations determined as PCR positive because of contamination with fungi and other bacteria. Further efforts are needed to demonstrate and separate viable organisms in an actual hospital environment.

We demonstrated that there is an overlap between the presence of *P. acanthamoeba* and *Acanthamoeba* in the hospital environment. *Acanthamoeba* spp. are distributed in a broad range of natural environments (2, 21, 22, 27, 29, 32) and have been classified into 15 different genotypes (T1 to T15) (13, 17, 19). It has been reported that the T4 genotype is frequently present in the natural environment and that 90% of isolates belong to the T4 genotype, which is the genotype most likely to exhibit strong virulence against humans (22, 26). Whether there is a possible variation that supports growth of *P. acanthamoeba* among *Acanthamoeba* genotypes remains to be seen. The elimination of *Acanthamoeba* may be important for preventing the emergence of *P. acanthamoeba* infections in hospitals.

In conclusion, this study demonstrated that the presence of *Acanthamoeba* had a significant effect on the presence of *P. acanthamoeba* in the hospital environment, suggesting that *P. acanthamoeba* could stably survive and spread in hospitals via free-living *Acanthamoeba* amoebae.

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