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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Running title: Prevalence of *P. acanthamoebae* and amoebae in hospitals

ABSTRACT

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 the 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 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 prevalence of *P. acanthamoebae* DNA in the first and second trials was 64.3% and 76%, respectively. The prevalence of *Acanthamoeba* DNA in the first and second trials was 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 was decreased among the samples from a moist area ($p = 0.382$). The *in vitro* experiment showed that without *Acanthamoeba*, *P. acanthamoebae* could not survive in dry conditions for 3 days (30°C) or 15 days (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; *Chlamydophila pneumoniae* is a causal agent of common respiratory infection and is also suspected of being involved in some chronic diseases, such as asthma and atherosclerosis (6), while *Chlamydia trachomatis* is 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 amoebae, *Acanthamoeba*, the most abundant genera of amoeba (1, 9, 11, 12). *P. acanthamoebae* and *S. negevensis* have been associated with lower respiratory tract infections (3, 27), and *W. chondrophila*, which was originally isolated from an aborted bovine fetus, is considered a potential abortigenic 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 polymerase chain reaction (PCR) in mononuclear cells of sputa and in bronchoalveolar lavage samples of 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 *Acanthamoeba* in hospitals and the survival of the bacteria in harsh conditions without *Acanthamoeba* remains unknown. We examined whether the prevalence of *P. acanthamoebae* 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 Bn_9 (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 infective progeny was determined according to the procedure described below. Free-living amoebae, *Acanthamoeba castellanii* C3 (ATCC 50739), was purchased from the American Type Culture Collection. Amoebae were maintained in PYG broth [0.75% (w/v) peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose] at 30°C (35). The infective progeny of *P. acanthamoebae* was determined a procedure known as the amoeba infectious units (AIU) assay, using co-culture 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 out patients; approximately 3,000 (per day), hospital patients; approximately 1,000 (per day)]** in a twelve-story building, located in **central area of** Sapporo **city**, 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 m^2 area on the floor or sink outlet with sterilized gauze (approximately 25 cm^2) 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% (v/v) Tween 80, and the suspension then centrifuged at 2,100 × g for 20 min. Pellets were resuspended in 200 µl PAS and then used for *P. acanthamoebae* culture and DNA extraction.

One half of the resuspended smear-pellet (100 µl) 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 µl of amoeba suspension containing 1×10^5 *A. castellanii* C3 cells in one well of a 96-well microplate and incubated for up to 10 days at 30°C in normal atmosphere. The microplate was read daily to determine the highest dilution of bacteria that led to amoebal lysis.

The DNA extraction was performed using UltraClean Soil DNA extraction kit (MBL, Corlsbad, CA, USA) according to the manufacturer's instructions. The primers used in PCR amplification were: 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* (191bp); 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 (423-551bp) (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* (216bp) (38). The primers for amplification of *P. acanthamoebae 16S rRNA* were designed based on GenBank cDNA

sequences (accession number NR026357) and using the program Primer 3 (http://frodo.wi.mit.edu/primer3/input.htm). To overcome inhibition of PCR amplification by humic acid, BSA was added to each reaction according to methods described 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 µl. The total reaction volume was 25 µl and consisted of 200 µM each dNTP, 10 µM BSA, 1× commercial reaction buffer and 0.625 U *Taq* DNA polymerase (New England Biolabs, Herts, UK). The thermal cycling profile involved an initial denaturation step at 94°C for 10 min followed by 35 cycles, each of 30 s denaturation at 94°C; 30 s annealing at 60°C for *P. acanthamoebae 16S rRNA*, 60°C for *Acanthamoeba* species *18S rRNA*, 52°C for bacterial *16S rRNA*; and 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, Korea). As a quality control for each PCR, diluted DNA extracted from *P. acanthamoebae* Bn_9 or *A. castellanii* C3 strains were 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 was performed using Genetyx-Mac software (version 10.1) and the Neighbor-Joining method in MEGA software (version 4). The detection limit of the PCR for *P. acanthamoebae 16S rRNA* and *Acanthamoeba 18S rRNA* was examined by using DNA extracted from the sterilized gauze moistened with PAS that had been spiked with defined numbers of *P. acanthamoebae* and *Acanthamoeba*, respectively. The detection limit of the PCR targeted to *P. acanthamoebae* was 10^2 AIU and 10 cells for *Acanthamoeba*

Procedure for monitoring bacterial viability is as below. A bacterial solution of 100 µl containing approximately 10^7-10^8 AIU prepared with PAS was placed into wells of a 24-well plate with (moist conditions) or without (dry conditions) 900 µl PAS, and the plates incubated for up to 28 days at 15°C or 30°C in normal atmosphere. At 0, 3, 7, 15 and 28 days after inoculation, the supernatant in each well was collected and centrifuged at 3,500 × 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 µl PAS and this was then transferred to a centrifuge tube. The resulting bacterial pellet was resuspended in 100 µl PAS. The infective progeny as a marker of bacterial viability in the solution was 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 (Molecular Probes, Eugene, OR, USA), according to the

manufacturer's instructions.

Correlation between the frequency of *P. acanthamoebae* and that of *Acanthamoeba* spp. was analyzed by Fisher's exact test. The influence of floor level on the prevalence of both organisms was also analyzed by a two-way ANOVA test. Comparison of bacterial numbers in the *in vitro* experiment was assessed by an unpaired *t* test. A *p* value of less than 0.05 was considered significant.

Prevalence of *P. acanthamoebae* and *Acanthamoeba* in a hospital environment. In total, ninety smear samples were collected from a hospital environment and assessed. The smear samples were randomly obtained from the floor and sink outlets. Fig. 1A shows a representative image of PCR amplification for *P. acanthamoebae*. The presence of amplified target genes was confirmed by direct sequencing with homology analysis. The DNA sequences obtained from *P. acanthamoebae* amplification products were mostly identical to those of previously reported *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 prevalence of *P. acanthamoebae* DNA in the first and second trials was 64.3% and 76%, respectively. The prevalence of *Acanthamoeba* DNA in the first and second trials was 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

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 or dry conditions. As shown in Fig. 3B, the numbers of *P. acanthamoebae* in moist conditions, regardless of culture temperature, was stably maintained for up to 28 days. The numbers of *P. acanthamoebae* grown in dry conditions rapidly decreased, with the bacterial numbers at 30°C more rapidly declining 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 the free-living amoebae, *Acanthamoeba*, 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*, 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* prevalence and survival due to the interaction of *P. acanthamoebae* with *Acanthamoeba* in the actual 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 non-culturable 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 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 prevalence of *P. acanthamoebae* and *Acanthamoeba* was very high in the hospital environment and there was a significant correlation between the prevalence of *P. acanthamoebae* and *Acanthamoeba*, how these organisms came to be inside a hospital still remains unknown. Interestingly, the prevalence of both organisms statistically changed depending on floor level, with the prevalence on lower floor levels much higher than at upper levels. It was also confirmed that the prevalence of both organisms in a total of 75 soil samples collected from **public parks (75 places) located in approximately 10 km square area around the hospital, which located in central area of Sapporo city,** was 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 when compared to 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 a *Acanthamoeba*

host. Meanwhile, we could not isolate *P. acanthamoebae* using amoebal-coculture system, but Thomas *et al*., has reported that *Chlamydia*-related organism in the 200 samples obtained from water system could be detected using amoebal-coculture method with an amoebae strain *A. castellanii* ATCC 30010C (38). Although the exact reason of contradiction between our results and their findings remain unknown, it is possible that usage of different amoebal strain is associated with these results. In addition, as mentioned above, since to prevent DNA contamination the preparation of the PCR mixture was performed in a separate room, 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 bacteria'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 when compared to moist conditions, suggesting that the protection and the survival of *P. acanthamoebae* through *Acanthamoeba* in the hospital environment would be limited, particularly in dry conditions. In contrast, *P. acanthamoebae* grown under the 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 *C. pneumoniae* and *C. trachomatis* and environmental the chlamydiae *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 should be needed to demonstrate and separate viable organisms in actual hospital environment.**

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

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

ACKNOWLEDGMNTS

This study was supported in part by grants-in aid for scientific research from KAKENHI, the Suhara Memorial Foundation, the Akiyama Foundation, and a research grant from the Institute for Fermentation, Osaka, Japan.

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Figure legends

FIG. 1. Detection and analysis of *P. acanthamoebae* DNA in smear samples obtained from a hospital environment. (A) Representative results of agarose gel electrophoresis of *P. acanthamoebae* PCR products amplified from smear samples. (B) Alignment analysis of four *P. acanthamoebae* amplicons (sample numbers 5, 8, 9 and 51) with previously reported *Parachlamydiaceae* sequences was performed using Genetyx-Mac software (version 10.1). Asterisk represents conserved sequences in all *P. acanthamoebae* PCR amplicons. Dots and unmarked positions represent a base mismatch and an additional base in the alignment, respectively. (C) Construction of a phylogenetic tree for *P. acanthamoebae* amplicons (sample numbers 5, 8, 9, and 51) with previously reported *Parachlamydiaceae* sequences was performed using the Neighbor-Joining method in MEGA software (version 4). Parenthesis, strain name. The accession numbers assigned by DDBJ was as follows: sample 5, uncultured *Parachlamydia* sp. (HUHP)-5 (AB565471); sample 8, HUHP-8 (AB565472); sample 9, HUHP-9 (AB565473); sample 51, HUHP-51 (AB565474).

FIG. 2. Influence of floor level on the prevalence of *P. acanthamoebae* (A) and *Acanthamoeba* (B). * indicates statistically significant.

FIG. 3. Representative images of LIVE/DEAD staining in *P. acanthamoebae* (A) and number 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, × 400.** (B) A bacterial solution of 100 µl containing approximately 10^7–10^8 AIU was prepared with PAS inoculated into the wells of a 24-well plate with (moist conditions) or without (dry conditions) 900 µl of PAS, and incubated for up to 28 days at 15°C or 30°C in normal atmosphere. The infective progeny as a marker of bacterial viability in the solution was determined using the AIU assay. The data represent the average AIU counts ± SD. * indicates $p < 0.05$.

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

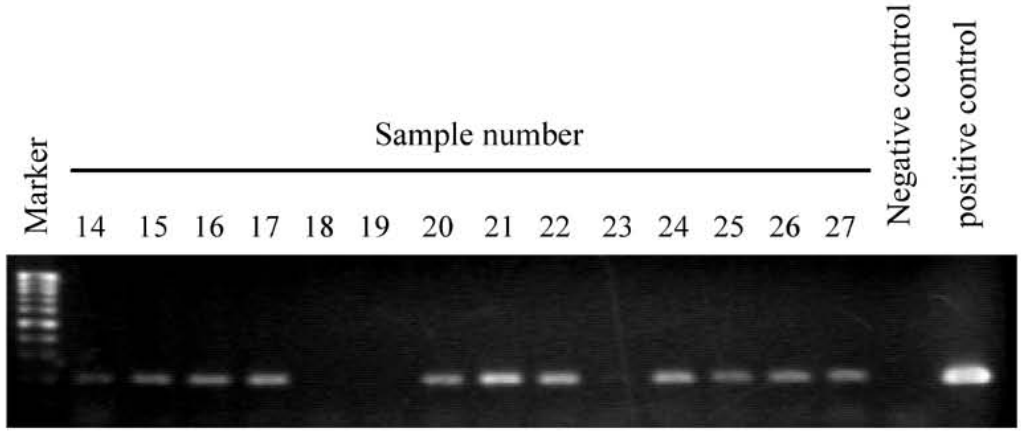
Prevalence of *Acanthamoeba* spp. DNA on different sampling period and site		Prevalence of *P. acanthamoebae* DNA		*p* value[a]
		Positive	Negative	
Sampling period				
1st trial: 2007 Nov.-2008 Mar.	Positive (*n* =	18 (64.3%)	7 (29.2%)	*p*=0.011 (S)[b]
	Negative (*n* =	10 (35.7%)	17 (70.8%)	
2nd trial: 2008 Oct.-2009 Feb.	Positive (*n* =	19 (76%)	5 (38.4%)	*p*=0.022 (S)
	Negative (*n* =	6 (24%)	8 (61.6%)	
Sampling site				
Sink outlet (*n*=34)[d] (moisture condition)	Positive (*n* =	6 (42.9%)	5 (25%)	*p*=0.273 (NS)[c]
	Negative (*n* =	8 (57.1%)	15 (75)	
Floor (*n*=56)[d] (dry condition)	Positive (*n* =	32 (82.1%)	7 (41.2%)	*p*=0.002 (S)
	Negative (*n* =	7 (17.9%)	10 (58.8%)	

[a]Correlation between the frequency of *P. acanthamoebae* and that of *Acantamoebae* was analyzed by Fisher's exact test.
[b]Significance.

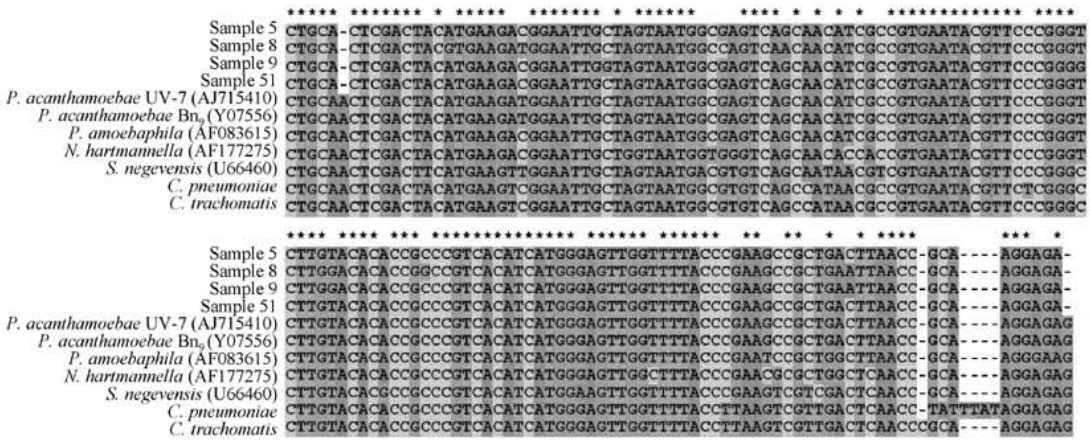
[c]Not significance.
[d]Mixed 1st trial with 2nd trial.

FIG 1.

A


Marker
Sample number
14 15 16 17 18 19 20 21 22 23 24 25 26 27
Negative control
positive control

B


Sample 5
Sample 8
Sample 9
Sample 51
P. acanthamoebae UV-7 (AJ715410)
P. acanthamoebae Bn9 (Y07556)
P. amoebaphila (AF083615)
N. hartmannella (AF177275)
S. negevensis (U66460)
C. pneumoniae
C. trachomatis

C

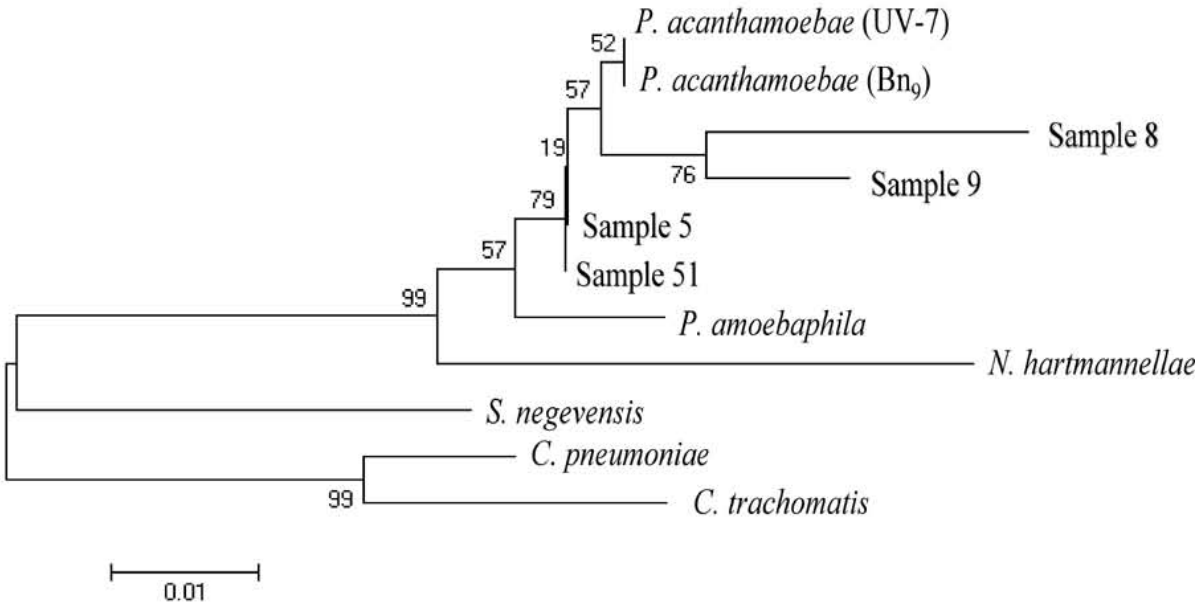
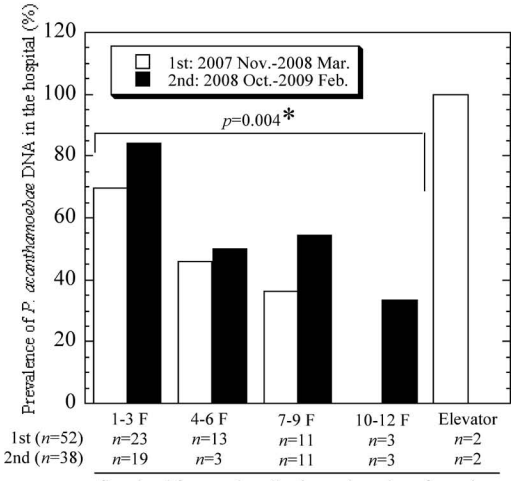

P. acanthamoebae (UV-7)
P. acanthamoebae (Bn9)
Sample 8
Sample 9
Sample 5
Sample 51
P. amoebaphila
N. hartmannellae
S. negevensis
C. pneumoniae
C. trachomatis
52
57
19
76
79
57
99
99
0.01

FIG 2.

A


Prevalence of P. acanthamoebae DNA in the hospital (%)
120
100
80
60
40
20
0
1st: 2007 Nov.-2008 Mar.
2nd: 2008 Oct.-2009 Feb.
p=0.004*
1-3 F
4-6 F
7-9 F
10-12 F
Elevator
1st (n=52)
2nd (n=38)
n=23
n=19
n=13
n=3
n=11
n=11
n=3
n=3
n=2
n=2
floor level for sample collection and number of sample

B

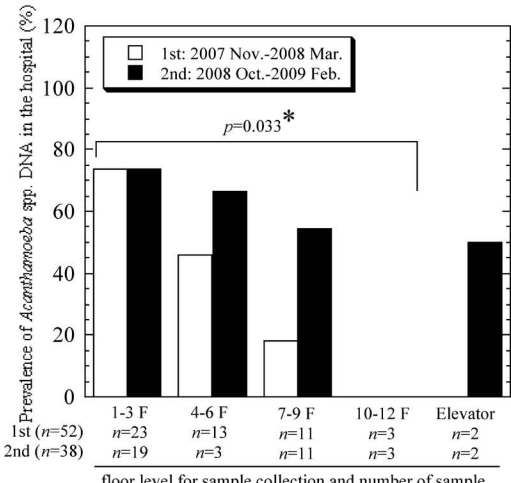
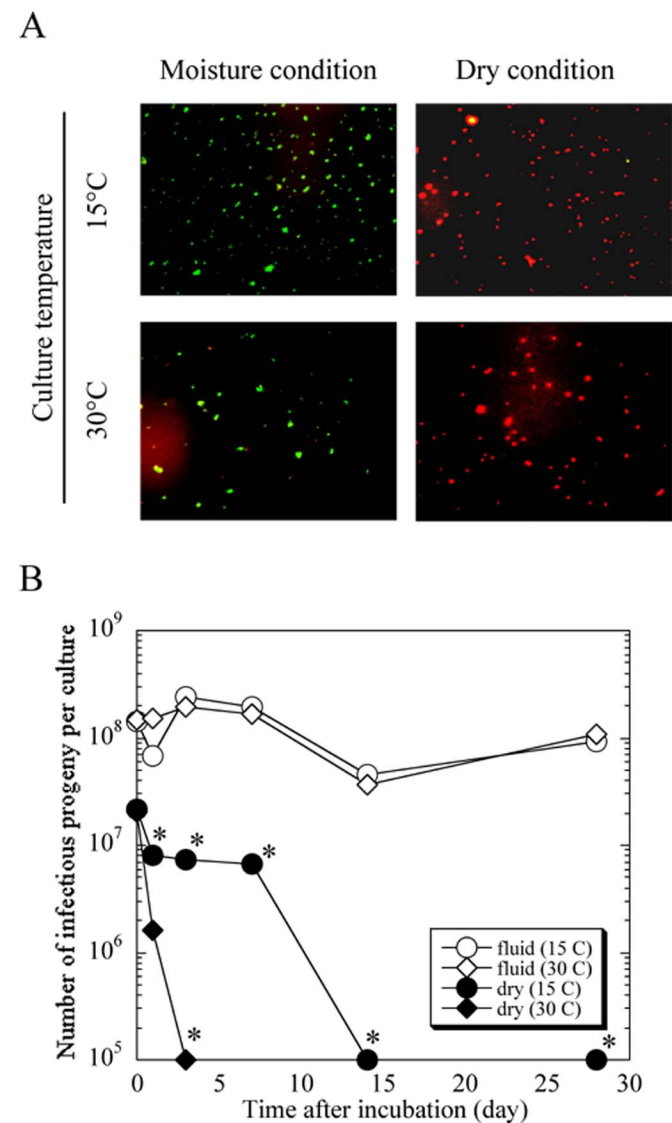

Prevalence of Acanthamoeba spp. DNA in the hospital (%)
120
100
80
60
40
20
0
1st: 2007 Nov.-2008 Mar.
2nd: 2008 Oct.-2009 Feb.
p=0.033*
1-3 F
4-6 F
7-9 F
10-12 F
Elevator
1st (n=52)
2nd (n=38)
n=23
n=19
n=13
n=3
n=11
n=11
n=3
n=3
n=2
n=2
floor level for sample collection and number of sample

FIG 3.


A
Moisture condition
Dry condition
Culture temperature
15°C
30°C
B
Number of infectious progeny per culture
10^9
10^8
10^7
10^6
10^5
fluid (15 C)
fluid (30 C)
dry (15 C)
dry (30 C)
0
5
10
15
20
25
30
Time after incubation (day)