Long-term survival of *Naegleria polaris* from Antarctica after 10 years storage at 4 °C

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Abstract

A free-living amoeba, *Naegleria* is ubiquitously distributed in various natural environments. Since some *Naegleria* spp. are exclusively distributed in the Arctic and sub-Antarctic regions, we hypothesized that the amoeba may be useful to determine long-term survival of *Naegleria* in laboratory conditions at 4 °C. Here, we show long-term survival of an amoeba, *N. polaris* isolated from a sediment sample, which was collected from Antarctica 10 years ago, and since stored at 4 °C. The sample was put on non-nutrient agar plates with heat-killed *Escherichia coli*, and then the plate was incubated at 4, 15, or 30 °C. Motile amoebae were seen only when the plate was incubated at 15 °C. The sequencing of ribosomal DNA including internal transcribed spacers (ITS)1, 5.8S rDNA, and ITS2 region revealed the amoebae to be *N. polaris*, which is exclusively distributed in the Arctic and sub-Antarctic regions. Scanning electron microscopic observation showed that no typical sucker-like structure was seen on the surface of *N. polaris*, but the cysts were similar to those of *N. fowleri*. Thus, our result shows, for the first time, that *N. polaris* can survive after 10 years storage at 4 °C. This finding may help us understand the still undescribed effects of environmental samples on viability of amoebae.

Keywords

amoebae, *Naegleria*, Antarctica, survival, long-term storage

Introduction

Equipped with beneficial capacities for adaptation, free-living amoebae (e.g. *Thecamoeba*, *Vahlkampfia*, *Hartmannella*, *Acanthamoeba*, or *Naegleria*) are ubiquitously distributed in natural environments such as soil, river water, pond water, and dust in the air. It is therefore believed that several amoebal species can survive even in extreme environments such as the Arctic or Antarctica (Brown et al. 1982; De Jonckheere 2006; Dillon et al. 1968; Tyml et al. 2016). In fact, some reports identified novel *Naegleria* spp. using ribosomal DNA sequencing of samples from the Arctic and sub-Antarctic regions (De Jonckheere 2006; Tyml et al. 2016). Since the genus *Naegleria* includes *N. fowleri*, which is a public health concern causing primary amoebic meningoencephalitis in human, with a high mortality rate (Schuster and Visvesvara 2004; Visvesvara et al. 2007), it is extremely important for us to understand the ecology of amoebae by assessing their viability in various environments. In fact, although limited, *in vitro* studies showing the long-term survival of amoebae in test tubes have already been reported. Specifically, nonpathogenic *N. gruberi* could survive in Page's amoeba saline (PAS) for 24 months at room temperature, for 14 months at 4 °C, or even for 8 months at -15 °C (Gupta and Das 1999). Similarly, pathogenic *N. fowleri* was stable for 17–22 months at room temperature (Biddick et al. 1984). Thus, the findings support the ability of *Naegleria* to survive for a long time in artificial laboratory conditions such as in PAS. However, because of the ubiquitous distribution of amoebae, the possibility of accidental contamination of these experiments with local amoebae cannot be ruled out. Meanwhile, a recent study showed that some *Naegleria* spp. such as *N. polaris* are exclusively distributed in the Arctic and sub-Antarctic regions (De Jonckheere 2014). Therefore, if *Naegleria*, found in only Antarcrtica, is

isolated from samples in Japan, we may clearly determine long-term survival of *Naegleria* in laboratory conditions without any concerns of contamination.

In this present study, *N. polaris* was isolated and identified from a sediment sample, which was collected from the Antarctic region 10 years ago and was immediately brought to Japan. As a result, we show here long-term survival of *N. polaris* after 10 years storage at 4 °C.

Materials and Methods

Samples

Three muddy sediment samples were collected from Oyako-ike, Abi-ike, or Maruwan o-ike Lakes on the Soya coast located in east Antarctica from December 2005 to January 2006. The samples were brought back to the Institute of Low Temperature Science, Hokkaido University, Japan and stored at 4 °C until use. The sample tubes were tightly sealed with Parafilm to avoid evaporation and contamination. In 2016, approximately 0.1 g of each sample was put on the center of a non-nutrient agar (NNA) plate with heat-killed *Escherichia coli* as described previously (Matsuo et al. 2010). Then, the plates were incubated at 4, 15, or 30 °C in humid conditions. After two weeks, the plates were observed carefully, using light microscopy, to identify amoebae that had emerged from the sample spot. The NNA agar block containing motile amoebae was put on freshly prepared NNA with *E. coli* and kept at the same temperature.

PCR

Amoebal DNA was extracted with the LaboPass Tissue Mini Kit (Cosmogenetech, Daejeon, Korea). PCR was conducted with primers for sequences of amoebal 18S

rDNA (forward1, 5′-CCA GCT CCA ATA GCG TAT ATT-3′; forward2, 5′-CCA GCT CCA AGA GTG TAT ATT-3′; reverse, 5′-GTT GAG TCG AAT TAA GCC GC-3′) (Thomas et al. 2006) or for *Naegleria* 5.8S rDNA with adjacent regions, referred to as internal transcribed spacers (ITS) 1 and ITS2 (forward, 5’-AAC CTG CGT AGG GAT CAT TT-3’; reverse, 5’-TTT CCT CCC CTT ATT AAT AT-3’) (De Jonckheere 2011). The first primers (18S rDNA) were used in order to identify up to genus, while the second primers (5.8S rDNA with adjacent regions ITS1 and 2) were used in order to identify up to species. After electrophoresis, the PCR products were purified using the FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan) and submitted to Macrogen Japan (Kyoto, Japan) for direct sequencing. The sequence was analyzed with NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) or BioEdit software version 7.2.5 (Hall 1999). The accession numbers identified in this study were as follows: LC318418 (18S rDNA partial sequence) and LC318419 (ITS1, 5.8S rDNA, and ITS2 region). A phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei 1987) in MEGA software version 7 (Kumar et al. 2016). The sequences of *Naegleria* spp. found in the Arctic or Antarctica (*N. polaris*, AM157657; *N. neopolaris*, AM157658; *N. arctica*, AM157659; *N. neoantarctica.* AM157662; *N. antarctica*, AJ566628) (De Jonckheere 2004; De Jonckheere 2006) and *N. fowleri* AR12 (X96564) (De Jonckheere 1998) as an outgroup are used.

Scanning electron microscopy (SEM)

Agar blocks containing amoebae were fixed with 2.5 % (v/v) glutaraldehyde in phosphate-buffered saline (pH 7.4) at 4 °C, and subsequently soaked in 2 % (w/v) osmium tetroxide for 1 h at 4 °C. The samples were then dehydrated in ethanol,

freeze-dried, and coated with osmium using a plasma osmium coater. Samples were analyzed using a Hitachi S-4800 SEM (Hitachi, Tokyo, Japan).

Results and Discussion

Although it is well known that free-living amoebae can robustly survive in a wide range of natural environments, it was an unknown factor for these amoebas whether they can survive for long periods of time, such as 10 years, in the laboratory condition. We therefore attempted to isolate amoebae from samples stored at 4 °C, were brought to Japan after being collected at three sites in Antarctica 10 years ago. Consequently, we found that a free-living amoeba identified as *N. polaris*, that exclusively inhabits the Arctic or Antarctic Circle (De Jonckheere 2014), can survive robustly even in a sample tube with poor nutrients at 4 °C for 10 years.

Since temperature is recognized as a fundamental factor that determines the growth rate of amoebae (De Jonckheere 2006), it was necessary to first determine the optimal temperature to successfully allow motility of amoebae from the sample collected from the Antarctic, an extremely cold environment. Therefore, the experiments described herein were performed at three distinct temperatures, 4, 15, and 30 °C. No motile amoebae were seen on plates cultured at 4 or 30° C. In contrast, motile amoebae were seen in a sample collected at Lake Oyako-ike, when incubated on an NNA plate with heat-killed *E. coli* at 15 °C for two weeks. After the initial 2-week incubation, the motile amoebae were isolated under a microscope and then maintained at the same temperature (15 °C). To confirm an optimal temperature for motile amoebae, a solution of isolated amoebae (5 μl) was again put on the center of an NNA plate with heat-killed *E. coli* at 4, 15, 30, or 37°C for a week. As expected, the amoebae moved

from the spots only when incubated at 15 °C (Figure 1). Although it was reported that *N. polaris* found in the therapeutic geothermal water sources in Iran could be grown at 30°C (Latifi et sl. 2017), this result indicates that the amoebae have adapted to a narrow temperature range, around 15°C, which corresponds to the highest temperature reached in the Antarctic summer (Peck et al. 2006). Although it is not a protist, the growth of the rod-shape bacterium *Hymenobacter nivism*, also isolated from Antarctica, has also been observed only at 15 °C (Kojima et al. 2016), supporting our data. Meanwhile, the average temperature, measured at the Showa station near the Soya coast, is -19.4 °C (August) to -0.7 °C (January) according to the Japan Meteorological Agency (http://www.jma.go.jp/jma/index.html). It is likely that the active growth state of amoebae inhabiting such a cold district is extremely limited.

We then performed molecular analysis of the isolated amoebae to determine their taxonomic classification. First, we amplified the extracted DNA of the amoebae with a primer set specific to an amoebal 18S rDNA region followed by the identification of the DNA sequences using a BLAST search, which can determine taxonomy to a genus level from a broad range of free-living amoebae. As a result, the amoebae were assigned to a genus, *Naegleria* (data not shown; E value = 0.0). Since the 18S rDNA sequences are not suitable for determining a species level classification of *Naegleria* (De Jonckheere 2006; De Jonckheere 2011), we then amplified the ITS region consisting of the ITS1, 5.8S rDNA, and ITS2 regions situated between the 18S and 28S rDNA, followed by direct sequencing and construction of a phylogenetic tree. As a result, the phylogenetic tree revealed that the amoebal sequence clustered into the same clade of *N. polaris and N. neopolaris* (Figure 2a). Analysis of the sequence length of the ITS regions in our *Naegleria* sp. revealed lengths of 36 bp for ITS1, 175 bp for 5.8S

rDNA, and 297 bp for ITS2, identical to those of *N. polaris* and *N. neopolaris* (Figure 2b). Substitutions of 8 base pairs in the ITS2 region of *N. neopolaris* (Figure 2c) compared with the sequence from our sample led us to conclude that the amoebae identified from the Antarctic sample were *N. polaris*.

N. polaris was first isolated in the Arctic (Spitzbergen and Greenland) and sub-Antarctic (Ile de la Possession) (De Jonckheere 2006). Several further studies also reported the isolation of *N. polaris*, however still exclusively in polar regions (De Jonckheere 2014). Thus, at present, since *N. polaris* is not thought to be naturally present in Asia, including Japan, we can exclude the possibility of the amoebae in our samples being present because of contamination. Therefore, we concluded that *N. polaris* could survive in a sediment sample for 10 years of storage at 4 °C.

Sucker-like structures, named amoebastome were first described in pathogenic *N. fowleri*, but not non-pathogenic *N. gruberi* (John et al. 1984; Marciano-Cabral and John 1983). These structures are involved in phagocytosis, including the ingestion of cultured cells, and are consequently thought to be a virulent factor of the amoebae (Marciano-Cabral and John 1983). The structure can also be found on the surface of *Acanthamoeba* (Diaz et al. 1991), which is a causative agent of amoebic keratitis or granulomatous amoebic encephalitis (Schuster and Visvesvara 2004; Visvesvara et al. 2007). To determine whether the structure was present on our amoebae, we visualized surface structures of *N. polaris* using SEM. As a result, two distinguishable forms, trophozoites and cysts, were clearly observed (Figure 3a: “trophozoite”, b: “cyst”). Trophozoite amoebae exhibited a small, polymorphic stoma-like structure (Figure 3a, arrowhead). Furthermore, evidence of food present in the stoma-like structure is observed (Figure 3c). However, the typical sucker-like structure was not found on the

surface of *N. polaris* trophozoites. In contrast, cysts showed more round cells of relatively uniform size with largely smooth surface structure (Figure 3b). Consistent with a previous study (Lastovica 1974), ruffled structures were also seen on the surface of cysts (Figure 3b, arrows), however the influence of fixation on the process during SEM sample preparations could not be excluded. Meanwhile, the morphological traits of the cyst were similar to those of *N. fowleri* (Lastovica 1974), supporting the amoebae belonging to the genus *Naegleria*.

In conclusion, we have demonstrated here, for the first time, that *N. polaris* can survive in an environmental sample of 4 °C, for 10 years of storage. This finding may help us understand the yet undescribed effects of environmental samples on viability of amoebae when they are storage in laboratory for a long period of time.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure 1 Temperature-dependent amoebal motility on NNA.

(a-d) NNA plates with heat-killed *E. coli* incubated at 4 °C (a), 15 °C (b), 30 °C (c), or 37 °C (d). The images were captured at a magnification of x40. (e) Enlarged image at a different field of NNA at 15 °C (x200). Arrowheads show motile amoebae.

Figure 2 Molecular identification of *Naegleria* spp. in Antarctica.

(a) A phylogenetic tree of 5.8S rDNA with adjacent regions (ITS1 and ITS2) of *Naegleria* was constructed using the Neighbor-Joining method (Saitou and Nei 1987) in MEGA software version 7 (Kumar et al. 2016). The number shows percentage calculated using the bootstrap test (1,000 replicates). Arrowhead shows *Naegleria* sp. isolated from Antarctica in this study. (b) Sequence length of ITS1, 5.8S rDNA, and ITS2 region of *Naegleria* spp. (c) Multiple alignments of ITS1, 5.8S rDNA, and ITS2 region of *Naegleria* spp.

Figure 3 SEM observation of *N. polaris*.

(a) Trophozoites. Arrowhead shows a stoma-like structure. (b) Cysts. Arrows show ruffled structure on the surface of cysts. Scale = 10 μm. (c) Enlarged image of the stoma-like structure seen in trophozoites.

302 Fig. 1

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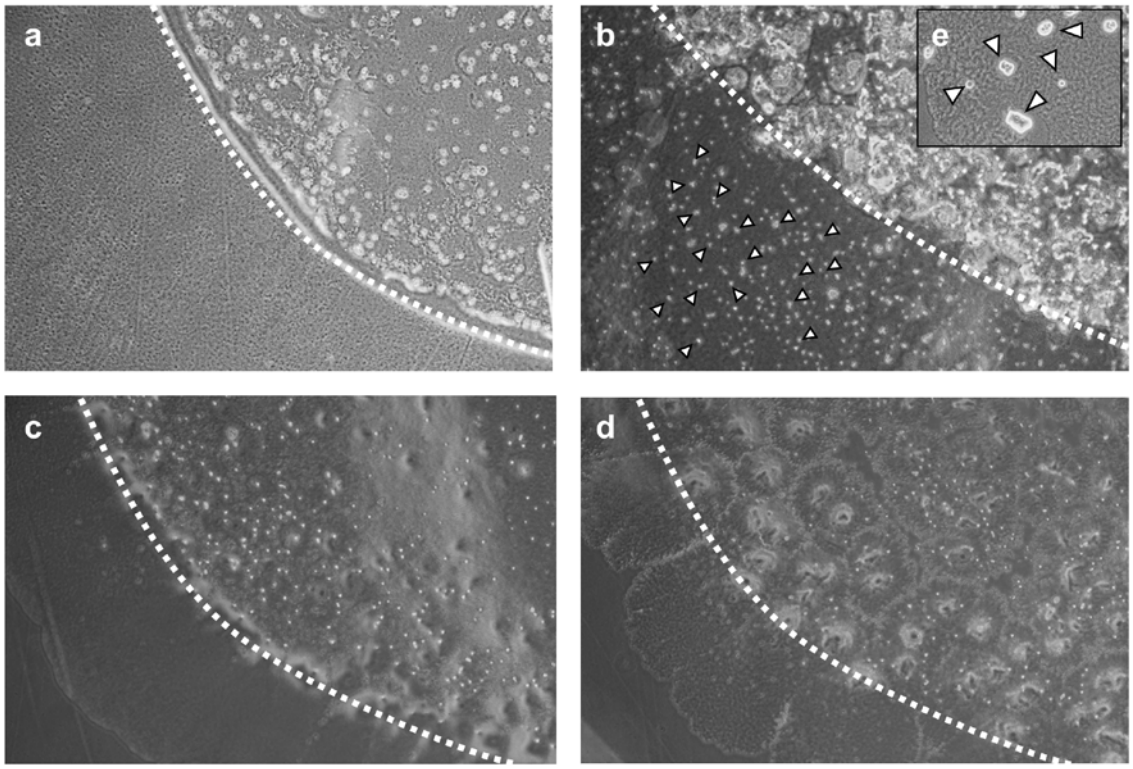

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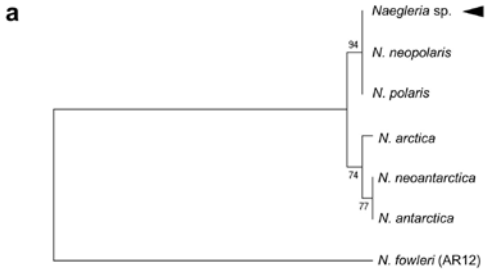
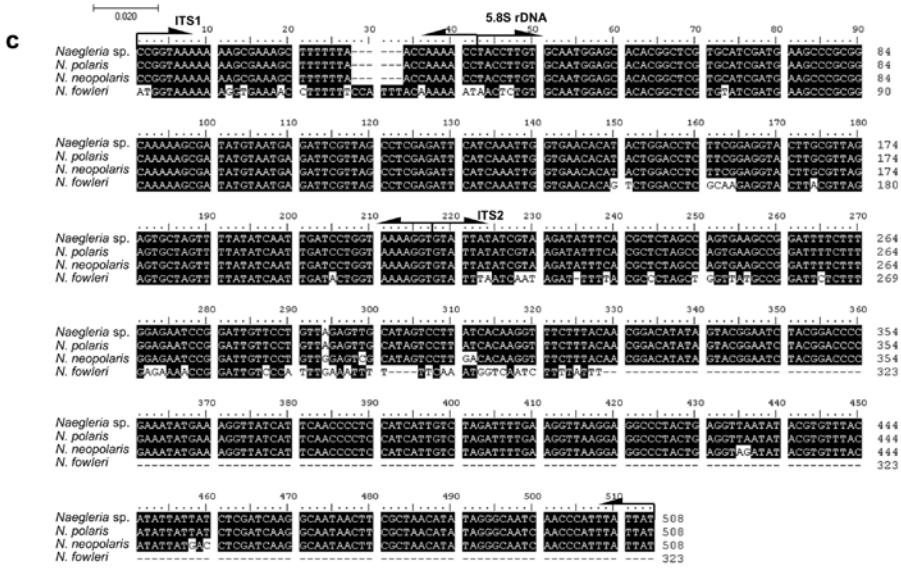
Fig. 2



b

Species	ITS1	5.8S rDNA	ITS2	References
Naegleria sp.	36	175	297	this study
N. polaris	36	175	297	De Jonckheere (2006)
N. neopolaris	36	175	297	De Jonckheere (2006)
N. arctica	36	175	454	De Jonckheere (2006)
N. neoantarctica	36	175	440	De Jonckheere (2006)
N. antarctica	36	175	439	De Jonckheere (2004)
N. fowleri AR12	42	175	106	De Jonckheere (1998)



318 Fig. 3

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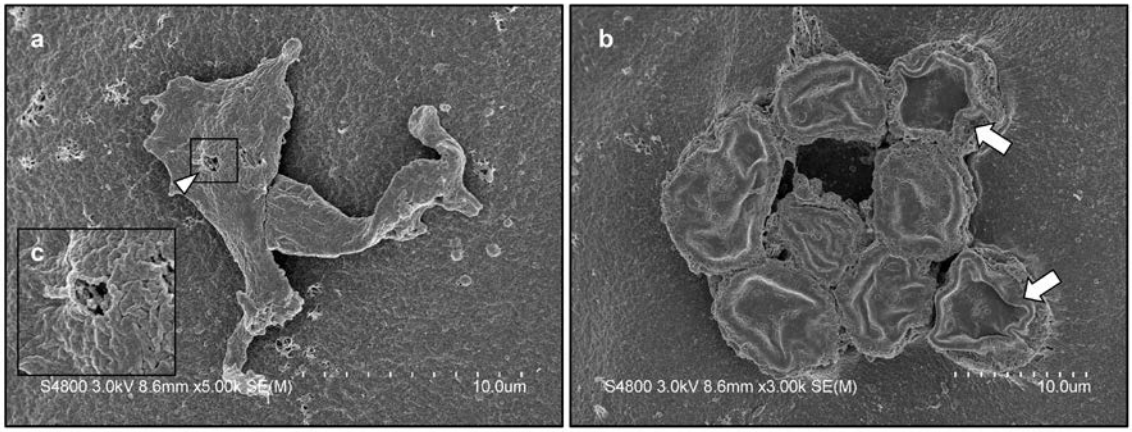

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