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Citation	Parasitology research, 117(3), 937-941 https://doi.org/10.1007/s00436-018-5779-9
Issue Date	2018-03
Doc URL	http://hdl.handle.net/2115/73419
Rights	The final publication is available at link.springer.com
Type	article (author version)
File Information	Parasitol Res. 117(3)_937-941.pdf



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1 **Long-term survival of *Naegleria polaris* from Antarctica**

2 **after 10 years storage at 4 °C**

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22

23 **Abstract**

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

40

41 **Keywords**

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

43

44 **Introduction**

45 Equipped with beneficial capacities for adaptation, free-living amoebae (e.g.
46 *Thecamoeba*, *Vahlkampfia*, *Hartmannella*, *Acanthamoeba*, or *Naegleria*) are
47 ubiquitously distributed in natural environments such as soil, river water, pond water,
48 and dust in the air. It is therefore believed that several amoebal species can survive even
49 in extreme environments such as the Arctic or Antarctica (Brown et al. 1982; De
50 Jonckheere 2006; Dillon et al. 1968; Tyml et al. 2016). In fact, some reports identified
51 novel *Naegleria* spp. using ribosomal DNA sequencing of samples from the Arctic and
52 sub-Antarctic regions (De Jonckheere 2006; Tyml et al. 2016). Since the genus
53 *Naegleria* includes *N. fowleri*, which is a public health concern causing primary
54 amoebic meningoencephalitis in human, with a high mortality rate (Schuster and
55 Visvesvara 2004; Visvesvara et al. 2007), it is extremely important for us to understand
56 the ecology of amoebae by assessing their viability in various environments. In fact,
57 although limited, *in vitro* studies showing the long-term survival of amoebae in test
58 tubes have already been reported. Specifically, nonpathogenic *N. gruberi* could survive
59 in Page's amoeba saline (PAS) for 24 months at room temperature, for 14 months at
60 4 °C, or even for 8 months at -15 °C (Gupta and Das 1999). Similarly, pathogenic *N.*
61 *fowleri* was stable for 17–22 months at room temperature (Biddick et al. 1984). Thus,
62 the findings support the ability of *Naegleria* to survive for a long time in artificial
63 laboratory conditions such as in PAS. However, because of the ubiquitous distribution
64 of amoebae, the possibility of accidental contamination of these experiments with local
65 amoebae cannot be ruled out. Meanwhile, a recent study showed that some *Naegleria*
66 spp. such as *N. polaris* are exclusively distributed in the Arctic and sub-Antarctic
67 regions (De Jonckheere 2014). Therefore, if *Naegleria*, found in only Antarctica, is

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

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

74

75 **Materials and Methods**

76 **Samples**

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

88

89 **PCR**

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

92 rDNA (forward1, 5'-CCA GCT CCA ATA GCG TAT ATT-3'; forward2, 5'-CCA GCT
93 CCA AGA GTG TAT ATT-3'; reverse, 5'-GTT GAG TCG AAT TAA GCC GC-3')
94 (Thomas et al. 2006) or for *Naegleria* 5.8S rDNA with adjacent regions, referred to as
95 internal transcribed spacers (ITS) 1 and ITS2 (forward, 5'-AAC CTG CGT AGG GAT
96 CAT TT-3'; reverse, 5'-TTT CCT CCC CTT ATT AAT AT-3') (De Jonckheere 2011).
97 The first primers (18S rDNA) were used in order to identify up to genus, while the
98 second primers (5.8S rDNA with adjacent regions ITS1 and 2) were used in order to
99 identify up to species. After electrophoresis, the PCR products were purified using the
100 FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan) and submitted to
101 Macrogen Japan (Kyoto, Japan) for direct sequencing. The sequence was analyzed with
102 NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) or BioEdit software version
103 7.2.5 (Hall 1999). The accession numbers identified in this study were as follows:
104 LC318418 (18S rDNA partial sequence) and LC318419 (ITS1, 5.8S rDNA, and ITS2
105 region). A phylogenetic tree was constructed using the Neighbor-Joining method (Saitou
106 and Nei 1987) in MEGA software version 7 (Kumar et al. 2016). The sequences of
107 *Naegleria* spp. found in the Arctic or Antarctica (*N. polaris*, AM157657; *N. neopolaris*,
108 AM157658; *N. arctica*, AM157659; *N. neoantarctica*. AM157662; *N. antarctica*,
109 AJ566628) (De Jonckheere 2004; De Jonckheere 2006) and *N. fowleri* AR12 (X96564)
110 (De Jonckheere 1998) as an outgroup are used.

111

112 **Scanning electron microscopy (SEM)**

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

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

118

119 **Results and Discussion**

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

128 Since temperature is recognized as a fundamental factor that determines the
129 growth rate of amoebae (De Jonckheere 2006), it was necessary to first determine the
130 optimal temperature to successfully allow motility of amoebae from the sample
131 collected from the Antarctic, an extremely cold environment. Therefore, the
132 experiments described herein were performed at three distinct temperatures, 4, 15, and
133 30 °C. No motile amoebae were seen on plates cultured at 4 or 30° C. In contrast, motile
134 amoebae were seen in a sample collected at Lake Oyako-ike, when incubated on an
135 NNA plate with heat-killed *E. coli* at 15 °C for two weeks. After the initial 2-week
136 incubation, the motile amoebae were isolated under a microscope and then maintained
137 at the same temperature (15 °C). To confirm an optimal temperature for motile amoebae,
138 a solution of isolated amoebae (5 µl) was again put on the center of an NNA plate with
139 heat-killed *E. coli* at 4, 15, 30, or 37°C for a week. As expected, the amoebae moved

140 from the spots only when incubated at 15 °C (Figure 1). Although it was reported that *N.*
141 *polaris* found in the therapeutic geothermal water sources in Iran could be grown at
142 30°C (Latifi et al. 2017), this result indicates that the amoebae have adapted to a narrow
143 temperature range, around 15°C, which corresponds to the highest temperature reached
144 in the Antarctic summer (Peck et al. 2006). Although it is not a protist, the growth of the
145 rod-shape bacterium *Hymenobacter nivism*, also isolated from Antarctica, has also been
146 observed only at 15 °C (Kojima et al. 2016), supporting our data. Meanwhile, the
147 average temperature, measured at the Showa station near the Soya coast, is -19.4 °C
148 (August) to -0.7 °C (January) according to the Japan Meteorological Agency
149 (<http://www.jma.go.jp/jma/index.html>). It is likely that the active growth state of
150 amoebae inhabiting such a cold district is extremely limited.

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

164 rDNA, and 297 bp for ITS2, identical to those of *N. polaris* and *N. neopolaris* (Figure
165 2b). Substitutions of 8 base pairs in the ITS2 region of *N. neopolaris* (Figure 2c)
166 compared with the sequence from our sample led us to conclude that the amoebae
167 identified from the Antarctic sample were *N. polaris*.

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

175 Sucker-like structures, named amoebastome were first described in pathogenic
176 *N. fowleri*, but not non-pathogenic *N. gruberi* (John et al. 1984; Marciano-Cabral and
177 John 1983). These structures are involved in phagocytosis, including the ingestion of
178 cultured cells, and are consequently thought to be a virulent factor of the amoebae
179 (Marciano-Cabral and John 1983). The structure can also be found on the surface of
180 *Acanthamoeba* (Diaz et al. 1991), which is a causative agent of amoebic keratitis or
181 granulomatous amoebic encephalitis (Schuster and Visvesvara 2004; Visvesvara et al.
182 2007). To determine whether the structure was present on our amoebae, we visualized
183 surface structures of *N. polaris* using SEM. As a result, two distinguishable forms,
184 trophozoites and cysts, were clearly observed (Figure 3a: “trophozoite”, b: “cyst”).
185 Trophozoite amoebae exhibited a small, polymorphic stoma-like structure (Figure 3a,
186 arrowhead). Furthermore, evidence of food present in the stoma-like structure is
187 observed (Figure 3c). However, the typical sucker-like structure was not found on the

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

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

199

200 **Acknowledgment**

201 The authors would like to thank the members of the 47th Japan Antarctica Research
202 Expedition for their logistic assistance and Y. Takano (Japan Agency for Marine-Earth
203 Science and Technology) for his support. We also thank Colleen Elso, PhD, from Edanz
204 Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

205

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

207

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- 279
- 280
- 281

282 **Figure 1 Temperature-dependent amoebal motility on NNA.**

283 (a-d) NNA plates with heat-killed *E. coli* incubated at 4 °C (a), 15 °C (b), 30 °C (c), or
284 37 °C (d). The images were captured at a magnification of x40. (e) Enlarged image at a
285 different field of NNA at 15 °C (x200). Arrowheads show motile amoebae.

286

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

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

295

296 **Figure 3 SEM observation of *N. polaris*.**

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

300

301

302 Fig. 1

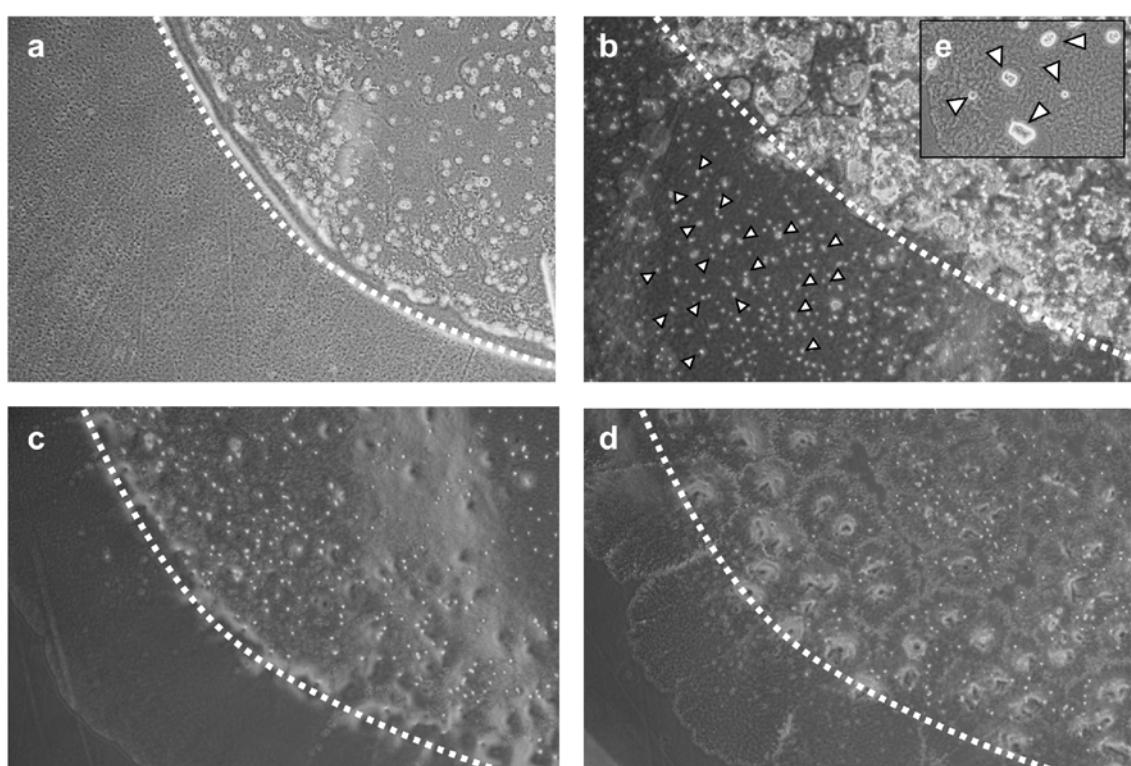
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310 Fig. 2

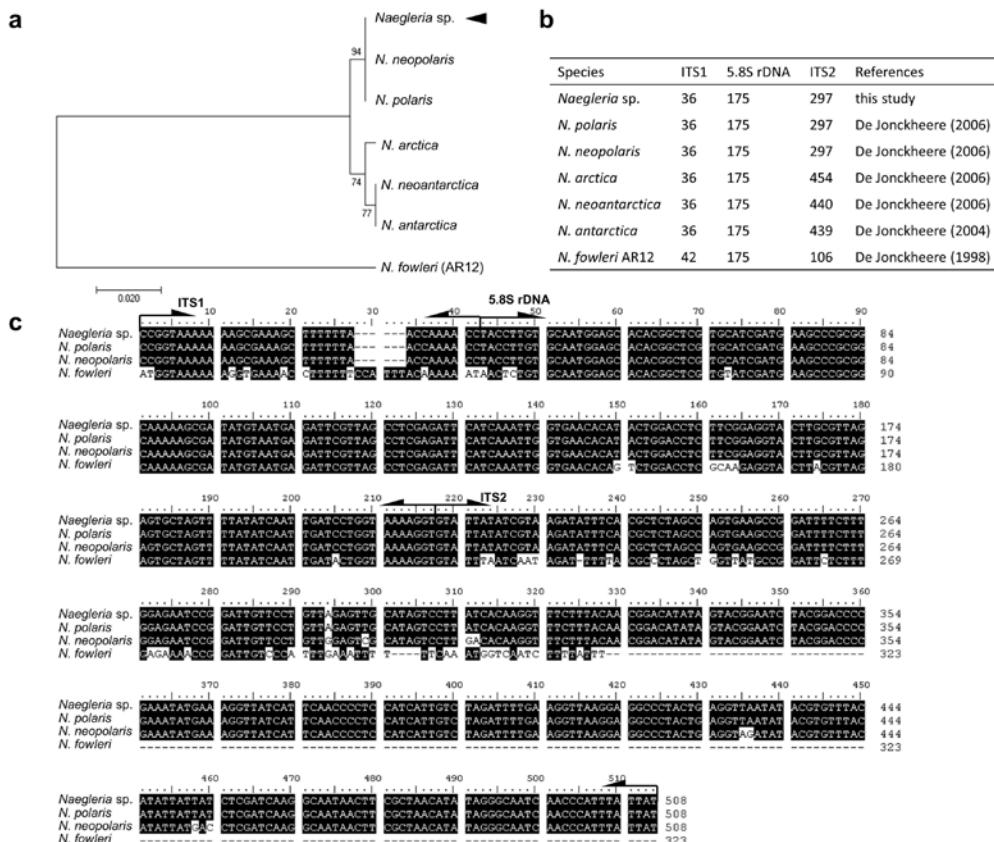
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318 Fig. 3

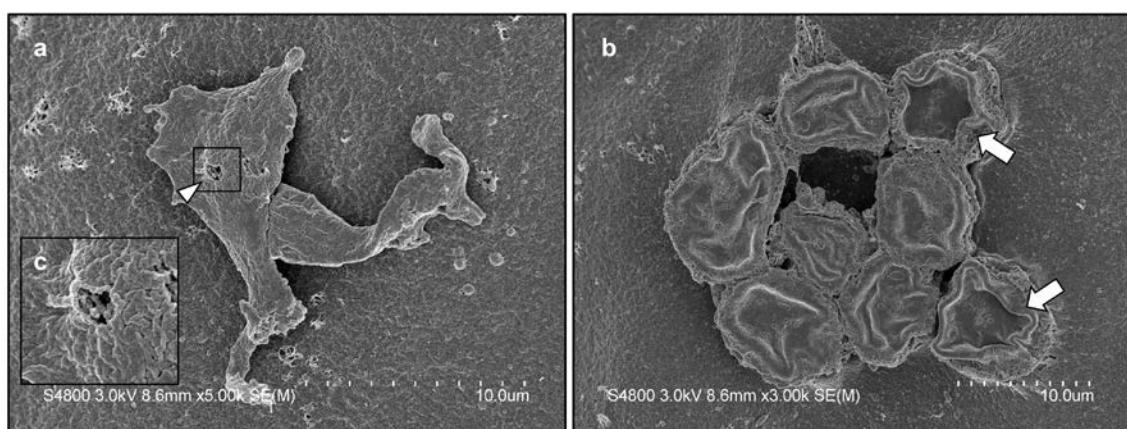
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