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First evidence of free-living *Naegleria* species in recreational lakes of Alberta, Canada

Rafik Dey [a,*], Elena Dlusskaya [b], Mariem Oloroso[a] and Nicholas J. Ashbolt [c]

[a] School of Public Health, University of Alberta, Edmonton, Canada
[b] Faculty of Agricultural, Life & Environmental Sciences, University of Alberta, Edmonton, Canada
[c] Faculty of Science and Engineering, Southern Cross University, Gold Coast, Qld, Australia
*Corresponding author. E-mail: rafik@ualberta.ca

RD, 0000-0002-4532-1751; ED, 0000-0003-4027-9221; NJA, 0000-0002-3853-0096

ABSTRACT

Rising temperatures are increasing environmental habitats for thermotolerant pathogens, such as the so-called 'brain-eating amoeba', *Naegleria fowleri*. To the best of our knowledge, however, *Naegleria* species have not been reported in environmental water sources in Canada. We surveyed popular recreational lakes in Alberta, Canada during the summer bathing period to determine the presence or absence of *Naegleria* species. While *N. fowleri* was not isolated in this study, we identified other thermotolerant species, including *Naegleria pagei*, *Naegleria gruberi*, *Naegleria jejuensis* and *Naegleria fultoni* using culture-based methods, hence indicating the potential conditions to support *N. fowleri*. Ongoing monitoring and examination of water for pathogenic amoebae is recommended in order to assist in the public health management of water sources.

Key words: amoebae, Canadian lakes, climate change, *Legionella*, *Naegleria* spp., waterborne pathogens

HIGHLIGHTS

- Survey of *Naegleria* species in recreational lakes in Alberta, Canada.
- First evidence of thermotolerant Naegleria species detection in Canada.
- Climate change and northern expansion of *Naegleria* species.

INTRODUCTION

Canada's climate has changed and is projected to continue to do so over the coming decades. Annual average temperatures over the Canadian landmass have increased by 1.7 °C and the north has warmed on average by 2.3 °C since 1948 (Bush & Lemmen 2019).

Recently, western provinces of Canada recorded temperatures exceeding 40 °C with a new Canadian temperature record of 49.6 °C registered in the village of Lytton (World Weather Attribution 2021). The increase in average temperature across Canada could allow water temperatures to become more suitable for the growth of thermotolerant pathogens such as members of the free-living amoebae (FLA).

Amoebae belonging to the genera *Naegleria* are potentially pathogenic to humans and commonly found in warm freshwater environments such as lakes, groundwaters and thermal springs. Exposures (such as via forced nasal entry) with these amoebae may result in neurological infections. The best example is *Naegleria fowleri*, 'the brain-eating amoeba', the causal agent of primary amoebic meningoencephalitis (PAM), which is an acute and rapidly fatal infection of the central nervous system (CNS) (Marciano-Cabral 1988). Northern hemisphere evidence suggests that climate change may expand the natural habitat of *N. fowleri* further north (Gompf & Garcia 2019; Maciver *et al.* 2020; Gharpure *et al.* 2021).

To the best of our knowledge, no reports on *Naegleria* or other FLA species have been reported in the environmental water sources of Canada. As a scoping study, our aim was to evaluate the presence of *Naegleria* spp. in different recreational lakes across the province of Alberta, Canada.

MATERIALS AND METHODS

Isolation and culture

Water samples were collected during the summer period from five different recreational lakes in the province of Alberta (Lac La Biche, Golden Sheaf Park, Lessard Lake, Wabamun Lake Provincial Park and Sylvan Lake) using 250-mL bottles (Fisherbrand™ 3001410008). Water samples were filtered using a vacuum manifold system with a 0.22-μm filter (MF-Millipore® Millipore, USA). Filters were then cultured inverted onto 2% non-nutrient agar (NNA) plates supplemented with a thin film of viable *Escherichia coli* (ATCC25922) as a food source for the amoebae at 30 °C and were monitored daily under an inverted light microscope to detect the growth of amoebae.

The identification of *Naegleria* species was based on the morphological features of trophozoites, cysts and the flagellate transformation test (Pussard & Pons 1977; Page 1988; de Jonckheere 2006). The flagellate transformation was induced by suspending the trophozoites in 1 mL of distilled water at 37 °C for 30–60 min.

For a pure amoebae culture and further investigations, we established isolates that showed the typical *Naegleria* morphology in axenic cultures in Nunc™ 25-cm^2 tissue culture flasks (ThermoFisher Scientific, Canada) containing 5 mL of Serum Casein Glucose Yeast Extract Medium (SCGYEM) ATCC medium 1021 at 37 °C with 10% of fetal calf serum (de Jonckheere & van de Voorde 1977).

PCR and DNA sequencing

Amoebae were harvested from culture flasks and DNA extraction was performed by using the DNeasy Blood & Tissue Kit™ (Qiagen, Canada). Two sets of primers were used for the identification of *Naegleria*: (i) *Naegleria* genus-specific (5′-GAACCTGCGTAGGGATCATTT-3′ and 5′-TTTCTTTTCCTCCCCTTATTA-3′) designed from the rDNA ITS regions as described previously (Pélandakis *et al.* 2000), (ii) *N. fowleri* species-specific (5′-TTCCGAACCCACTCAATAAA-3′ and 5′-TTCCGAACCCTTAAAACCTC-3′) targeting a single-copy DNA region (Régoudis & Pélandakis 2016).

Reaction conditions for both sets were: 5 min at 94 °C, followed by 40 cycles at 94 °C for 1 min, 55 °C for 30 s, 72 °C for 2 min, and a final extension for 10 min at 72 °C. The resulting PCR products were purified using the QIAquick PCR purification kit™ (Qiagen, Canada) and sent to The Applied Genomics Core (University of Alberta) for sequencing and species identification based on sequence homology analysis by comparison to the available amoebae species sequences in the Genbank database.

Transmission electron microscopy

Axenic cultures of amoebae were fixed at room temperature with 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences 15960). Samples were submitted for processing at the imaging core at University of Alberta, faculty of biological sciences.

Sectioned and carbon-coated samples were observed with a Hitachi H-7650 transmission electron microscope (TEM).

RESULTS AND DISCUSSION

All selected amoebic cultures were morphologically positive for the *Naegleria* genus. The trophozoite, cyst and flagellate stages are illustrated in Figure 1. This was subsequently confirmed by partial Sanger sequencing. As shown in Table 1, the sequencing results from the axenic cultures revealed the presence of four distinct *Naegleria* species: *Naegleria pagei*, *Naegleria gruberi*, *Naegleria jejuensis* and *Naegleria fultoni*.

Despite the absence of *N. fowleri*, all four identified *Naegleria*l isolates are thermotolerant (Kilvington & White 1985), suggesting that the environments examined could promote the proliferation of *N. fowleri*. For example, recent cases associated with *N. fowleri* have been reported in Minnesota (USA), which shares a 547-mile border with the Canadian provinces of Manitoba and Ontario (Gompf & Garcia 2019). It is therefore not surprising that pathogenic *Naegleria* species could be present in Canada due to increasing water temperatures driven by climate change.

Moreover, food sources are known to play an important role in *Naegleria* spp. growth and presence (Goudot *et al.* 2012; Morgan *et al.* 2016; Puzon *et al.* 2020). As bacterivores, previous studies have shown that amoebae including *Naegleria* spp. uses a range of bacteria, especially *Meiothermus* spp. and *E. coli* as the preferred prey (Kyle & Noblet 1985; Schuster 2002; Xue *et al.* 2018; Puzon *et al.* 2020). In fact, environmental growth of *E. coli* has been reported in recreational Great Lakes (Verhougstraete *et al.* 2010) and faecal contamination related to increasingly intense storm events is another consideration for increased *E. coli* levels (Whitman & Nevers 2008).

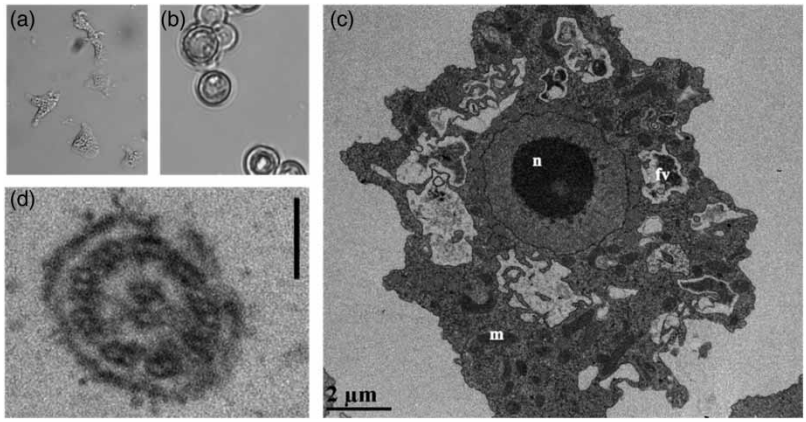

Figure 1 | Morphological characteristics of isolated *Naegleria gruberi* in axenic culture. Micrographs of (a) trophozoite (40×) and (b) cyst (100×) using a phase-contrast microscope (Leica CTR 4000). (c) Transmission electron microscopy showing a trophozoite of the isolated amoeba. Nucleus (n), mitochondria (m), food vacuole (fv). (d) Transverse section of the flagellum of the temporary flagellated stage. Scale bar = 100 nm.

Table 1 | Sequence homology of isolated *Naegleria* species

Isolate accession number	Closest phylogenetic species	Homology (%)	Reference strain accession number
OQ034612	*Naegleria pagei*	99.39	DQ768721
OQ034613	*Naegleria gruberi*	97.80	MG699123
OQ034614	*Naegleria jejuensis*	99.11	MF113400
OQ034615	*Naegleria fultoni*	95.82	DQ768719

Additionally, the isolated amoebae are known hosts of a temperature-selected pathogen *Legionella pneumophila*; a waterborne bacterium responsible for Legionnaires' disease in engineered environments, soils and organic-rich media (Barbaree *et al.* 1986; Huang & Hsu 2010; Zbikowska *et al.* 2013; Dupuy *et al.* 2016). Therefore, increased FLA presence in water sources represents a potential risk, enhanced through the carriage of amoeba-resisting pathogens such as *L. pneumophila* (Thomas *et al.* 2010). To the best of our knowledge, this is the first report on the occurrence of *Naegleria* species in Canadian recreational waters, consistent with the worldwide distribution of *Naegleria* spp. in water sources (Saberi *et al.* 2020), and adding further information on the identification of *Naegleria* species in North America. As surface water temperatures continue to rise with climate change and the availability of bacterial food source increases, further investigations should be conducted to better understand the distribution and microbial ecology of problematic *Naegleria* spp. for water quality management strategies to prevent future amoebic infections.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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