Acta Tropica 119 (2011) 114–118

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

# Survey of *Naegleria* from Taiwan recreational waters using culture enrichment combined with PCR

Shih-Wei Huang[a], Bing-Mu Hsu[b,*]

[a] Super Micro Mass Research and Technology Center, Cheng-Shiu University, Kaohsiung, Taiwan, ROC
[b] Department of Earth and Environmental Sciences, National Chung Cheng University, 168, University Rd, Min-Hsiung, Chiayi, Taiwan, ROC

ARTICLE INFO

*Article history:*
Received 27 August 2010
Received in revised form 27 April 2011
Accepted 28 April 2011
Available online 26 May 2011

*Keywords:*
*Naegleria*
Spring water
Stream water
Raw domestic water
Recreational water

ABSTRACT

*Naegleria* is a free-living amoeba. Pathogenic *Naegleria* may pose a health risk to people who come in contact with recreational waters. Here, we used *Naegleria* culture enrichment with PCR to identify the *Naegleria* species and investigated the distribution of *Naegleria* spp. in recreational waters including spring water, stream water and raw domestic water in central and southern Taiwan. In this study, *Naegleria* spp. were detected in 19 (17.8%) of the water samples. The occurrence of *Naegleria* in raw domestic water was 28.6%, higher than in stream water (14.7%) and in spring water (6.5%). The most frequently identified species exhibiting the closest phylogenetic relationships to the isolates were *N. australiensis* ($n = 4$) and *N. canariensis* ($n = 4$), followed by *N. clarki* ($n = 3$) and *N. philippinensis* ($n = 3$); *N. americana* ($n = 2$). *N. lovaniensis*, *N. dobsoni*, and *N. gruberi* were each detected once. The pathogenic species *N. fowleri* was not detected, probably due to the low incubation temperature; however, the isolates exhibiting the closest phylogenetic relationships to the pathogenic species in mice of PAM, *N. australiensis* and *N. philippinensis*, were found. Results of this survey suggest the distribution of *Naegleria* spp. excluding *N. fowleri* in recreational waters. It should be considered a potential threat for health associated with human activities in recreational waters.



## 1. Introduction

Free-living amoebae (FLA) are ubiquitous in soil and aquatic environments. They feed by phagocytosis, mainly on bacteria, fungi, and algae. Of the many hundreds of FLA species, *Naegleria* is the one of the predominant FLA and can be isolated in lakes, rivers, geothermal water, discharge from industrial plants, swimming pools and spas (Marciano-Cabral and Cabral, 2003; Hsu et al., 2009). More than 30 species of the genus *Naegleria*, an amoeba in the family *Vahlkampfiidae*, have been classified using molecular techniques (De Jonckheere, 2004). Among them, *Naegleria fowleri* is proved to be the only one species that is pathogenic to humans. *N. fowleri* is transmitted via the nasal mucosa and the olfactory nerve to the brain, resulting in primary amoebic meningoencephalitis (PAM), an acute and rapidly fatal disease of people following exposure to polluted water (Martinez and Visvesvara, 1997). The number of PAM cases has increased in recent years and almost 200 cases have been reported since 1965 (Visvesvara et al., 2007; Edagawa et al., 2009). Infections occur in children and young adults – age groups that are more energetic in aquatic activities and thus are likely to come into contact with amoebae in water (Visvesvara et al., 2007). *N. australiensis*, *N. italica* and *N. philippinensis* have displayed pathogenicity in few laboratory animals because their virulence is lower than that of *N. fowleri* (De Jonckheere, 2002; Schuster, 2002; Visvesvara et al., 2007).

The genus *Naegleria*, like *Acanthamoeba* and *Hartmannella*, can present an extra threat to human health because they can support the growth and dispersal of facultative intracellular bacteria, including pathogens and other microorganisms. *N. clarki* and other *Naegleria* species have been described to harbor endocytobionts (Walochnik et al., 2005). The most-studied *Naegleria* resistant bacterium is *Legionella*, which can survive and grow in *Naegleria*, and exit from them (Greub and Raoult, 2004; Declerck et al., 2007).

In addition to the raw drinking water, Taiwanese people frequently come in contact with raw water at recreational springs. Visiting hot springs is a very popular form of recreation. The water sources of hot springs include springs and streams. Some studies report *Naegleria* infestation in raw drinking water, spring water and stream water worldwide. John and Howard (1995) reported that *N. australiensis* and *N. fowleri* appeared in 38% and 18% of FLA isolate samples from Oklahoma ponds and streams. Sheehan et al. (2003) showed that the thermotolerant amoeba, *N. fowleri*, is not a transient organism, but thrives in Yellowstone and Grand Teton National Parks hot springs. Edagawa et al. (2009) found *N. australiensis*, a potentially pathogenic species, in tap water from Osaka, Japan.

* Corresponding author. Tel.: +886 5 2720411; fax: +886 5 2720807.
*E-mail address:* bmhsu@ccu.edu.tw (B.-M. Hsu).

0001-706X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2011.04.016

**Table 1**
Locations and descriptions of sampling sites.

| Sampling site | Latitude/longitude | Location of Taiwan | Water source | Number sampled |
|---|---|---|---|---|
| SA1 | 24°01′N, 121°11′E | Midland | Carbonate spring area | Spring: 7<br>Stream: 8 |
| SA 2 | 23°33′N, 120°55′E | Midland | Carbonate spring area | Spring: 5<br>Stream: 6 |
| SA 3 | 23°20′N, 120°29′E | The South | Mud spring area | Spring: 3<br>Stream: 10 |
| SA 4 | 23°07′N, 120°42′E | The South | Carbonate spring area | Spring: 4<br>Stream: 2 |
| SA 5 | 22°05′N, 120°45′E | The South | Carbonate spring area | Spring: 9<br>Stream: 5 |
| SA 6 | 22°41′N, 121°00′E | The South | Sodium bicarbonate spring area | Spring: 3<br>Stream: 3 |
| WTP1 | 22°39′12″N, 120°21′18″E | The South | River | 6 |
| WTP2 | 22°36′16″N, 120°25′58″E | The South | River | 6 |
| WTP3 | 22°35′27″N, 120°24′02″E | The South | River | 12 |
| WTP4 | 22°32′42″N, 120°23′31″E | The South | River | 12 |
| WTP5 | 22°40′48″N, 120°24′20″E | The South | River | 6 |

However, researchers have not yet studied the potential infestation of *Naegleria* in recreational waters in Taiwan. An investigation of the occurrence and distribution of *Naegleria* in local water is imperative because of its possible health implications. This study samples water from springs, streams, and domestic water sources in central and southern Taiwan. The aim of this study was to determine the presence of *Naegleria* in various water types and various recreational areas. Naegleriae were detected with microbial culture, combined with molecular methods that allow the taxonomic identification of *Naegleria* species. To extend our analysis, we collected other information, including the physical and microbiological water quality parameters.

## 2. Materials and methods

### 2.1. Study location and sampling procedures

A total of one hundred-seven recreational water samples including spring water, stream water and raw domestic water were collected between January, 2008 and September, 2009 from six spring recreational areas and five water supply plants in central and southern Taiwan (Table 1). The spring water samples were from nature spring including chloride spring water, weak alkaline carbonate spring water and weak alkaline sodium bicarbonate water, and the stream water samples were collected in the identical areas that the spring water sampled. The raw domestic water samples from water supply plants were pumped from rivers and lake in southern Taiwan. Each sample (about 2000 ml) was placed into a sterile bottle and transported to the laboratory for subsequent analysis.

### 2.2. Physical and microbiological parameters analysis

Water samples taken for heterotrophic bacteria and total coliforms were collected in 300 ml sampling bag (Nasco Whirl-Pak, USA). Heterotrophic bacteria were measured by the spread method. Total coliforms were measured by membrane filtration procedures with a differential medium described in the Standard Method for the Examination of Water and Wastewater (Methods 9222 B and D) (APHA, 1995). Water temperature and its pH were measured in situ using a portable pH meter (D-24E, Horiba Co., Japan). Turbidity was measured using a turbidimeter (HACH Co., Loveland, CO). The calculations of difference in the presence/absence of Naegleriae in terms of five water quality parameters were conducted using the STATISTICA software (StatSoft, Inc., USA).

### 2.3. Naegleria detection procedure

To survey of *Naegleria* in environmental water body, water samples were filtered through 45-mm diameter cellulose nitrate membranes (Pall, USA) with a pore size of 0.22 μm. 1 l of water was filtered through three to six cellulose nitrate membranes, which were subsequently inverted and placed onto 1.5% non-nutrient agar plates containing a lawn of *Escherichia coli*. The number of cellulose nitrate membranes used for each sample depended on the turbidity of the water sample. The plates were sealed and incubated at 28 °C for 14 days. The colonies on the plate were all transferred in PYG medium consisting of 2% proteose peptone, 0.2% yeast extract, and 0.1 M glucose (Visvesvara and Balamuth, 1975). The samples that yielded amoebae were harvested and suspended in sterile phosphate-buffered saline (PBS; 7.5 mM $Na_2HPO_4$, 3.3 mM $NaH_2PO_4$, 108 mM NaCl, pH 7.2). All experiments were conducted in triplicate. Each water sample treated with microbial culture was centrifuged to 300 μl at 9700 × *g*, 10 min. DNA extraction kit (Nucleospin® Tissue, Macherey-Nagel Inc., Germany) was used to extract total genomic DNA according to the kit manual and was then subjected to PCR reaction.

The PCR reaction solution was prepared with 5 μl of the DNA templates and 200 pmol primers together with PCR mixture to create a total volume of 50 μl. PCR mixture contained 5 μl 10× PCR buffer (20 mM $MgCl_2$), 1 μl dNTP mix (10 mM of each dNTP) and 0.5 μl VioTaqTM DNA polymerase (Viogene, 5 U/μl) and DNase-free deionized water. Negative DNA controls (template DNA replaced with distilled water), and positive controls (*Naegleria* ATCC 22758) and sample DNA were analyzed in triplicate during each PCR run. PCR assay primers, PCR cycling conditions, and the target gene segments used for detecting Naegleriae in this study are shown in Table 2.

Aliquots (5–10 μl) of each amplicon after PCR were mixed with 1–2 μl of loading buffer (10 mM EDTA, 10% glycerol, 0.015% bromophenol blue, 0.17% SDS) and separated on a 2.0% agarose gel. Products were visualized by ethidium bromide staining and imaged under UV light. After the amplification products were confirmed by gel electrophoresis and then were also analyzed by sequencing