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Identification and significance of *Naegleria fowleri* isolated from the hot spring which related to the first primary amebic meningoencephalitis (PAM) patient in Taiwan

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ABSTRACT

Naegleria fowleri can cause primary amoebic meningoencephalitis, a rapidly developing and highly lethal infectious disease. The first confirmed case of primary amoebic meningoencephalitis in Taiwan was reported in November 2011, in which the patient visited a thermal spring recreational area 1 week prior to hospitalisation. Water sampling was performed to verify the presence of *Naegleria* at the facility. According to our results, 32% and 20% of recreational water samples were contaminated with *Naegleria* spp. and *Acanthamoeba* spp., respectively. The genotypes of *Naegleria* identified at the hot spring included *N. fowleri*, *Naegleria australiensis* and *Naegleria lovaniensis*. Using PCR, it was determined that the strain of *N. fowleri* in one sample possessed the same genotype 2 as the clinical isolate. Thus, the thermal spring was suggested to be the likely source of infection. This is the first known instance of simultaneously isolating *N. fowleri* from both a patient as well as from a hot spring in Taiwan. Following this initial study, the pools at the thermal spring recreational area were drained, scrubbed and disinfected, and a follow-up study was performed 1 month later. *Naegleria fowleri* was not detected in follow-up testing; however, other *Naegleria* spp. were identified. We postulate that the biofilm in the waterlines may have provided a reservoir for free-living amoebae. The presence/absence of *Acanthamoeba* and *Naegleria* spp. did not differ significantly with any measured parameters related to water quality; however, a high percentage of the thermal water pool samples were contaminated with *Naegleria* or *Acanthamoeba*. Thus, amoebic contamination may present a serious threat to the health of humans who engage in leisure activities at thermal springs.

1. Introduction

Protozoan meningitis can be caused by *Naegleria fowleri*, *Balamuthia mandrillaris* and some species of *Acanthamoeba*. The fatal meningitis caused by *N. fowleri* is referred to as primary amebic meningoencephalitis (PAM). PAM infection mainly occurs via the nasal route, i.e., nasal mucosa contact with water containing *N. fowleri*. Early symptoms of PAM include headache, fever, nausea, vomiting and stiff neck. Without proper treatment, the disease progresses rapidly and usually causes death within 3–7 days (Craun et al., 2005; Petit et al., 2006). The first confirmed human infection by *N. fowleri* was reported by Fowler and Carter in 1965 (Fowler and Carter, 1965). Since then, approximately 235 PAM cases have been reported. Most of the PAM cases were children and approximately 5% of patients survived (Grate, 2006). Due to its high mortality rate, PAM is considered a serious problem for public health worldwide (Heggie, 2010; De Jonckheere, 2011). Besides *N. fowleri*, *Naegleria philippinensis* and *Naegleria italica* have been identified in human infection cases. Initially, *N. italica* was reported only in Italy, but more recently it has been found worldwide (De Jonckheere, 2002). Also, *N. philippinensis* was isolated from human CSF in 1984 (Matias, 1991). In contrast, other species of

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Naegleria, such as *Naegleria australiensis* and *Naegleria lovaniensis*, have not been identified in cases of human infection (Schuster, 2002).

Naegleria fowleri is able to survive in temperatures up to 45.8 °C, making the species especially adaptive to mammalian body temperature (Marciano-Cabral and Cabral, 2007). In the United States (US), hot springs were found to contain *N. fowleri* (Craun et al., 2005). There have been 120 PAM cases reported in North America and the cases were mainly in the southern states of the US and Mexico (De Jonckheere, 2011). In Europe, 24 confirmed PAM cases have been reported in the UK, Italy and Belgium (De Jonckheere, 2011). In Australia, there have been 19 PAM cases and more than half of the cases were from southern Australia (De Jonckheere, 2011). In Asia, 39 confirmed cases of PAM have been reported and most of the cases were from southern Asia, including Pakistan, India and Thailand (De Jonckheere, 2011). In Japan, *N. fowleri* was isolated from geothermal water and industrial cooling water near the home of a PAM patient but no direct association could be established (Izumiyama et al., 2003). In Taiwan, *Naegleria* and other free-living amoebae (FLA) were found in hot springs and surface waters, but *N. fowleri* was never found in the environment (Huang and Hsu, 2010b, 2011; Kao et al., 2012b).

The source and transmission route of *N. fowleri* has not been well characterised and there is no data regarding the occurrence of *N. fowleri* in hot springs in Taiwan. A reason for the lack of information was that human infections of protozoan meningitis have been rare in Taiwan. In November 2011, the Taiwan Center for Disease Control (CDC) Parasite Laboratory received a CSF sample from a suspected PAM patient. The patient died a few days later and the CSF specimen was found to contain DNA from *N. fowleri*, making it the first confirmed PAM case in Taiwan. The patient engaged in frequent leisure activities in a hot spring resort, thus waters at the resort were suspected to contain the pathogen. Water samples were taken from the facility to determine potential sources of infection and the facility was shut down for cleaning and disinfection before resuming normal operation.

This study was initially performed on request from the Taiwan CDC in response to the suspected (and later confirmed) PAM patient. One month after disinfection, a follow-up sampling study was also performed. The occurrences of *Naegleria* spp. and *Acanthamoeba* spp. in the water samples were evaluated. Several experimental methods were used simultaneously to increase detection efficiency and taxonomic identification was undertaken to identify species of the detected *Naegleria* and *Acanthamoeba*. The study results and their potential implications for public health are presented and discussed.

2. Materials and methods

2.1. Sample collection

Water samples were collected from a hot spring resort in Taiwan. Spring waters at the resort were from a natural hot spring and contained chloride, weak alkaline carbonate and weak alkaline sodium bicarbonate. In addition, stream water was used as the source of cold water at the resort. The initial sample collection was carried out on 30 November 2011, which was the day after the onset of hospitalisation for the suspected PAM patient. Water samples were collected from water reservoirs, personal pools, personal spa and wastewater. After water samples were collected, the resort was shut down by the health authority, and the facility was cleaned and disinfected as recommended by the health authority. In short, the cleaning and disinfection procedures included draining of all water reservoirs, pools and tubs, followed by surface scrubbing to remove any potential biofilms and disinfection with sodium hypochlorite. A follow-up sampling campaign was carried out on 29 December 2011 to determine the effectiveness of the cleaning and disinfection procedure.

For each sampling location, approximately 1 L water samples were collected and placed into two sterile bottles. The samples were stored at ambient temperature and analysed within 8 h. Each water sample was concentrated before diagnostic PCR analysis and genotype analysis was performed to identify *Naegleria* spp. and *Acanthamoeba* spp. In addition, physical water quality parameters were measured for each sample location at the time of sample collection and microbiological parameters were assessed for each sample location.

2.2. Detection of free-living amoebae (FLA)

Each water sample was filtered through 45 mm diameter cellulose nitrate membranes (Pall, USA) with a pore size of 0.22 μm. The filter was washed and DNA extraction was performed for FLA by PCR analysis. Additionally, *N. fowleri* Carter strain (ATCC 22758) was used as a positive control. The genotyping primer set, ITS1/ITS2, and diagnostic primer set, Nae3-For/Nae3-Rev, were, respectively, used to amplify a 400–453 bp fragment from the complete intergenic transcribed spacer (ITS) gene sequences of different genotypes of *Naegleria* and a 183 bp fragment of the 18S ssrRNA gene sequences of *N. fowleri*, *N. lovaniensis* and *Naegleria gruberi*. A summary of the primers and probes used in this study is presented in Table 1. Thermal cycling conditions for PCR analysis of *Naegleria* (Nae3 primer set) included 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, and 50 °C for 45 s. PCR analysis of the ITS region included 95 °C for 5 min, followed by 40 cycles of three steps, 95 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s, and a final extension of 72 °C for 10 min.

Detection and confirmation of *Acanthamoeba* was done via real-time PCR and genus-specific PCR with the DNA extract from each water sample. The diagnostic real-time PCR primer set included AcantF900/AcantR1100 and probe AcantP1000 to detect *Acanthamoeba* spp. (Table 1). The PCR mixture (20 μl) included 4 μl of 5× LightCycler TaqMan Master (Roche Diagnostics, Mannheim, Germany), 0.5 μM of each primer, 0.2 μM of probe and 5 μl of DNA in glass capillary tubes, and amplification was done on a LightCycler 2.0 instrument (Roche Diagnostics). Samples were rated as positive when the LightCycler software determined a crossing point in the quantification analysis. The genus-specific PCR primer set included JDP1/JDP2 to amplify a PCR product from the 18S rDNA stretch *Acanthamoeba*-specific amplimer (ASA) S1 of *Acanthamoeba* genotypes. Thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 62 °C for 45 s, 72 °C for 45 s, and a final extension of 72 °C for 10 min for the genus-specific PCR.

Following PCR, aliquots (5–10 μl) of each amplicon were mixed with 1–2 μl of loading buffer (10 mM EDTA, 10% glycerol, 0.015% bromophenol blue, 0.17% SDS) and separated on 2% agarose gel. The separation products were visualised by ethidium bromide staining and imaged under UV light. The amplification products were confirmed by gel electrophoresis and the genetic sequences were analysed using the Bio-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA), according to kit specifications. When directly sequencing PCR products yielded unreadable results, further cloning was performed by ligating the PCR product in T&A Cloning Vector (Real Biotech Co., Taiwan) and transforming it into *Escherichia coli* DH5α cells. The cloning procedure was conducted according to instructions provided by the manufacturer. Plasmid DNA was subsequently extracted from selected colonies and confirmed by PCR. For each cloned sample, approximately six colonies were selected for PCR confirmation and sequencing.