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Surveillance and evaluation of the infection risk of free-living amoebae and *Legionella* in different aquatic environments

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HIGHLIGHTS

- *Acanthamoeba* and *Naegleria* are prevalent in hot springs and raw drinking water.
- Most of the identified *Acanthamoeba* are potentially pathogenic.
- *Acanthamoeba* frequently coexist with higher level of environmental bacteria.
- *Legionella* frequently coexist with *H. vermiformis* in aquatic environments.

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ABSTRACT

Free-living amoebae (FLA) are ubiquitous in various aquatic environments. Several amoebae species are pathogenic and host other pathogens such as *Legionella*, but the presence of FLA and its parasites as well as the related infection risk are not well known. In this study, the presence of pathogenic FLA and *Legionella* in various water bodies was investigated. Water samples were collected from a river, intake areas of drinking water treatment plants, and recreational hot spring complexes in central and southern Taiwan. A total of 140 water samples were tested for the presence of *Acanthamoeba* spp., *Naegleria* spp., *Vermamoeba vermiformis*, and *Legionella*. In addition, phylogenetic characteristics and water quality parameters were also assessed. The pathogenic genotypes of FLA included *Acanthamoeba* T4 and *Naegleria australiensis*, and both were abundant in the hot spring water. In contrast, *Legionella pneumophila* was detected in different aquatic environments. Among the FLA assessed, *V. vermiformis* was most likely to coexist with *Legionella* spp. The total bacteria level was associated with the presence of FLA and *Legionella* especially in hot spring water. Taken together, FLA contamination in recreational hot springs and drinking water source warrants more attention on potential legionellosis and amoebae infections.

1. Introduction

In developing countries, waterborne diseases account for about 80% of infectious diseases and one-third of fatal infections (WHO, 2003). Among waterborne disease pathogens, free-living amoebae (FLA) are ubiquitous in natural water environments such as hot springs, rivers, lakes, and drinking water (Ettinger et al., 2003). The most frequently isolated environmental FLA include *Acanthamoeba*, *Naegleria*, and *Vermamoeba* (Martinez and Kasprzak, 1980; Seal et al., 1999). In earlier studies, nearly 80% of healthy individuals were found to have anti-*Acanthamoeba* antibodies, suggesting high likelihood of pathogen contact (Alizadeh et al., 2001; Chappell et al., 2001). Several genotypes of FLA, such as *Acanthamoeba* T4 and *Naegleria fowleri*, are opportunistic pathogens to animals and humans (Dykova et al., 1999; Frank and Bosch, 1972; Visvesvara et al., 2007a). *Acanthamoeba* could cause Acanthamoeba keratitis, which may lead to blindness if not properly treated (Jones et al., 1975). Brain infection of *Acanthamoeba* leads to highly fatal Granulomatous Amebic Encephalitis (GAE) (Jager and Stamm, 1972). Infection with *N. fowleri* could cause primary amoebic

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meningoencephalitis (PAM) which is likely to be fatal following brain invasion (Grate, 2006).

An important issue with FLA is their role as hosts of harmful bacteria. Several species of *Legionella* are known parasites of *Acanthamoeba* spp. (Adeleke et al., 2001; Drozanski, 1956; Drozanski, 1991; La Scola et al., 2001; Rowbotham, 1986). The genus *Legionella* includes 52 species and 71 serogroups, and at least 20 species have been associated with human diseases (Bargellini et al., 2011; Den Boer et al., 2008; Doleans et al., 2004; Yu et al., 2002). Among the pathogenic *Legionella*, *Legionella pneumophila* accounts for more than 80% of legionellosis cases, a respiratory infection with 20% fatality rate (Nazarian et al., 2008).

Legionella are known to adapt to different environments and survive under a wide range of temperatures (5–65 °C). They are found in surface waters, wetlands, and inadequately sterilized water distribution systems (Guillemet et al., 2010). Inhalation of contaminated water droplets is a major route of infection for *Legionella* (Hsu et al., 1984; Steinert et al., 2002). The presence of endosymbionts such as *Legionella* in *Acanthamoeba* has been found to mutually increase the pathogenicity and virulence of both pathogens (Cirillo et al., 1999; Fritsche et al., 1998). In addition, parasitic *Legionella* in amoebae are more resistant to environmental stresses such as disinfectants (Abu Kwaik et al., 1998). The amoebae hosts also enhance the ability of parasitic *Legionella* to invade epithelial cells or macrophages (Cirillo et al., 1994).

In Taiwan, about 100 cases of legionellosis are reported annually, according to Taiwan CDC. In 2009, the first confirmed case of GAE was reported in Taiwan (Sheng et al., 2009). Since these FLA-related waterborne diseases are most likely to be of environmental origin, assessment of FLA in different aquatic environments is crucial. In this study, the presence of FLA and *Legionella* in different water bodies was investigated. In addition to detection and speciation of FLA, the genotype of *Legionella* spp. was determined. Environmental conditions related to presence of FLA and parasitic *Legionella* are discussed, and this paper concludes with recommendations for future research needs.

2. Materials and methods

2.1. Sample collection and pretreatment

Water samples were collected from Puzih River (denoted as group A; see Fig. 4 for its geographic location in Taiwan), raw water intake area at five drinking water treatment facilities along Kaoping River (denoted as group B), and two hot spring recreation complexes in central and southern Taiwan (denoted as group C). Puzih River is an important water source for local agriculture, fish farming and industries. Kaoping River is the main source of drinking water supply for Kaohsiung City, the largest city in southern Taiwan. To facilitate microbe filtration and subsequent experiments, the hot spring water samples were mostly collected at the points with clear but not muddy water.

The sample collection took place between August, 2011 and April, 2012. For each water sample, about 2000 ml of water was taken into two sterile 1 l bottles and transported to the laboratory at 4 °C within 24 h. At each sampling site, three water quality parameters were measured on site, including pH level with a portable pH meter (D-24E, Horiba Co., Japan), water temperature with a thermometer, and turbidity with a turbidimeter (HACH Co., Loveland, CO, USA).

For each water sample, quantitative microbial parameters were assessed within 24 h after sample collection, including heterotrophic bacteria by spread method and total coliforms by membrane filtration, both followed by incubation on a differential medium as described in the Standard Method for the Examination of Water and Wastewater (Methods 9215 C and 9222 B) (APHA, 2005).

To concentrate microbes, a 1 l water sample was filtered through 45-mm diameter GN-6 material membranes (Pall, Mexico City, Mexico) with a pore size of 0.22 μm in a stainless steel filter holder. The membranes were then eluted with 100 ml of sterilized phosphate-buffered saline (PBS). The eluent was transferred into two conical centrifuge tubes (50 ml each) and centrifuged at 2600 $\times g$ for 30 min. For each centrifuged solution, the top supernatant fluid (about 47 ml) was discarded, and the remaining 2–3 ml concentrate was used for subsequent experiments.

2.2. FLA cultivation

The FLA cultivation procedure followed that described in an earlier study (Lorenzo-Morales et al., 2007). After filtration, the membranes were directly covered on NNA agar plates seeded with killed *Escherichia coli* (NNA-*E. coli*). The samples were incubated at 30 °C, 85% humidity for 3 to 4 days. Subsequently, the filters were removed and the plates were incubated for 3 to 4 more days. Finally, potential plaques were picked for DNA extraction and PCR analysis.

2.3. DNA extraction

Each sample concentrate or cultured colony was transferred to a new eppendorff tube. Subsequently, the DNA was extracted using MagNA Pure LC equipped with MagNA Pure LC DNA isolation kit III (Roche, Nutley, NJ, USA). The quality and quantity of extracted DNA were evaluated by a NanoDrop spectrometer (Thermo Scientific, Wilmington, DE, USA).

2.4. Conventional and real-time polymerase chain reaction (PCR)

For conventional PCR, 3 μl of derived extract containing at least 5 ng/μl DNA was added into a PCR mixture consisting of 10× PCR Buffer (with 20 mM $MgCl_2$), 10 mM dNTP Mix, 100 pmol each of the oligonucleotide primers, VioTaqTM DNA Polymerase (Viogene, Taipei, Taiwan), and DNase-free deionized water. The primers used in reactions for most strains of FLA and *Legionella* are summarized in Table 1. Each test result was confirmed by agarose electrophoresis. Sterilized ddH_2O was used as negative control, and clinically identified or purchased *Acanthamoeba*, *Naegleria* (ATCC 22758), *Vermamoeba* (ATCC50237), and *L. pneumophila* were used as positive controls.

In this study, real-time PCR was used to quantify specific microbes in each sample. To produce standard curves for each microbe, the PCR product of each tested microbe was cloned into yT&A vector (Yeasterm Biotech Corporation, Taipei, Taiwan). The recombinant plasmids were purified using HiYield™ plasmid mini kit (Real Biotech Corporation, Taipei, Taiwan), and quantified using a micro-spectrophotometer (CLUBIO CB-4500, Taiwan) at 260 nm in triplicate. The corresponding copy number was determined according to the prescribed equation (Whelan et al., 2003). The standard curve was derived by serial 10-fold dilutions of the recombinant vectors, and the regression coefficient was kept higher than 0.995 in each experiment. Real-time PCR (Taqman or SYBR Green) was performed using ABI StepOneTM Systems and recommended reagents (Applied Biosystems, Singapore). Finally, the quantity of target genes was determined by normalizing the derived threshold cycle value (Ct) to the standard curves.

2.5. Phylogenetic analysis

Phylogenetic analysis was conducted using MEGA version 4.0.2 (The Biodesign Institute, Tempe, AZ, USA). The phylogenetic trees were derived by the neighbor-joining method with 1000 bootstrap simulations.

2.6. Statistic analysis

Statistical analyses were performed on the presence of target microbes with the water quality parameters using STATISTICA version 6.0 (StatSoft, Inc., Boston, MA, USA).