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Surveillance of viable *Acanthamoeba* spp. and *Naegleria fowleri* in major water sources for tap water in Korea[§]

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한국 주요 상수원수에서의 가시아메바와 파울러자유아메바 조사

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The pathogenic free-living amoebas (FLAs), *Acanthamoeba* spp. and *Naegleria fowleri*, can cause fatal infections, including amoebic encephalitis. They are ubiquitously distributed in nature, including in diverse bodies of water. In order to survey *Acanthamoeba* spp. and *N. fowleri* in source water in Korea, we used culture-based real-time PCR to detect viable FLAs in 52 source water samples collected between July 2017 and December 2017. *Acanthamoeba* spp. and *N. fowleri* were detected in 42 samples (80.8%) and 6 samples (11.5%), respectively. *Acanthamoeba* spp. were detected at approximately the same frequency in all seasons, but *N. fowleri* was mainly detected in summer and autumn, with no *N. fowleri* detected in winter. These results demonstrate that these pathogenic FLAs, especially *N. fowleri*, which has caused deaths in the United States and China, are widely distributed in the Korean aquatic environment.

Keywords: free-living amoebas, pathogenic amoebas, protozoan parasites, real-time PCR, source water

Free-living amoebas (FLAs) are widely distributed in natural environments throughout the world (da Rocha-Azevedo *et al*., 2009). Among the many genera of free-living amoebas, four genera, *Acanthamoeba*, *Naegleria, Balamuthia*, and *Sappinia*, have been reported to cause diseases in humans (Schuster and Visvesvara, 2004; da Rocha-Azevedo *et al*., 2009). Several species of *Acanthamoeba* are pathogenic, and can cause serious amoebic keratitis in healthy people (Marciano-Cabral and Cabral, 2003). In addition, in immunodeficient people, *Acanthamoeba* spp. may cause the opportunistic infection granulomatous amoebic encephalitis (Siddiqui and Khan, 2012). *Naegleria fowleri*, widely known as the 'brain-eating amoeba', is commonly distributed in soil and warm water, including rivers, lakes, and hot springs (De Jonckheere, 2012). Among the more than 30 species of *Naegleria*, *N. fowleri* is the only recognized species that causes infections in humans (De Jonckheere, 2004; Visvesvara *et al*., 2007). Acute infection with *N. fowleri*, known as primary amoebic meningoencephalitis, can be fatal. This infection is appeared through exposure during aquatic activities (Martinez and Visvesvara, 1997).

These pathogenic FLAs are classified as pathogenic protozoa in the contaminants candidate list (CCL) of the U.S. Environmental

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Protection Agency (EPA). Additionally they are monitored and controlled to ensure drinking water safety. Unfortunately, in the Republic of Korea, there has been little surveillance of these pathogenic FLAs in aquatic environments including source water for water supplies (Jeong and Yu, 2005; Jung *et al*., 2008).

In addition to being ubiquitous and pathogenic, these FLAs are highly resistant to sterilization. This makes them a risk factor in treated drinking water. De Jonckheere and van de Voorde (1976) and Sarkar and Gerba (2012) reported that *Acanthamoeba* spp. are highly resistant to both chlorine and UV light, with a level of UV resistance even higher than that of *Cryptosporidium* oocysts. De Jonckheere and van de Voorde (1976) found that *N. fowleri* also showed high sterilization resistance, and Cursons *et al*. (1980) found that inactivation of cysts required exposure to a residual chorine concentration of 0.74 mg/L for 30 min. Thus, for the safety of drinking water in Korea, detection of these FLAs in source water is imperative.

For the detection of *Acanthamoeba* spp. and *N. fowleri*, specific molecular-based techniques have been developed, including PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (Mathers *et al*., 2000; Schroeder *et al*., 2001; Marciano-Cabral and Cabral, 2003; Qvarnstrom *et al*., 2006; Madarova *et al*., 2010; Yang *et al*., 2013; Derda *et al*., 2014; Mahittikorn *et al*., 2003). These molecular techniques are highly sensitive, but a major weakness is found in those methods which can not discriminate between living and dead FLAs.

In the present study, we designed new primers and probes for detecting *Acanthamoeba* spp. and *N. fowleri* based on the recently updated genome database of NCBI GenBank. In addition, we developed a cell culture-based real-time PCR method for specifically detecting viable *Acanthamoeba* spp. and *N. fowleri*. Using these newly developed real-time PCR-based techniques, 52 samples from water sources for tap water were surveyed in 2017 for the presence of viable cases of the two FLAs, *Acanthamoeba* spp. and *N. fowleri*.

Materials and Methods

Reference strains

Acanthamoeba castellanii (Douglas) Page (ATCC 30011) and *Naegleria fowleri* Carter (ATCC 30215) were purchased from the American Type Culture Collection (Manassas, VA, USA). To maintain both amoebas in their active states, the *A. castellanii* and *N. fowleri* were incubated at 30°C for 4~5 days in Nelson's medium (Qvarnstrom *et al*., 2006) and fresh water amoeba medium (ATCC Medium 997), respectively.

Environmental samples

A total of 52 samples were collected from source water for Korean tap water treatment, between July and December in 2017 (Fig. 1). Each sample consisted of one liter of water collected from a single sampling location. From each sample, 970 ml of the water was filtered using a polycarbonate filter (Isopore Membrane Filters, Merck Millipore Ltd.) with a pore size of 0.8 μm and the filter was then submerged in the remaining 30 ml of the water sample and vortexed vigorously (Ozcelik *et al.*, 2012; Moussa *et al.*, 2013). Then, the filter was removed, and the resuspended mixture was centrifuged at 1,500 × *g* for 20 min. The supernatant was then discarded, leaving a residue of 2 ml in the tube. Half of this residue, 1 ml of the concentrated sample, was then cultured on a non-nutrient agar (ATCC Medium 919) plate, prepared by pre-inoculating inactivated *Escherichia coli* KCTC 2441 (Korean Collection for Type Cultures, Jeongeup, Republic of Korea) at 30°C for

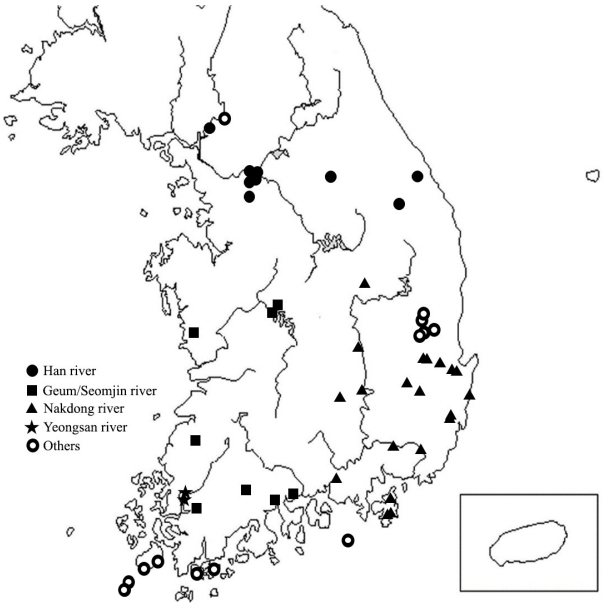

Fig. 1. Sampling locations of water sources in Korea.

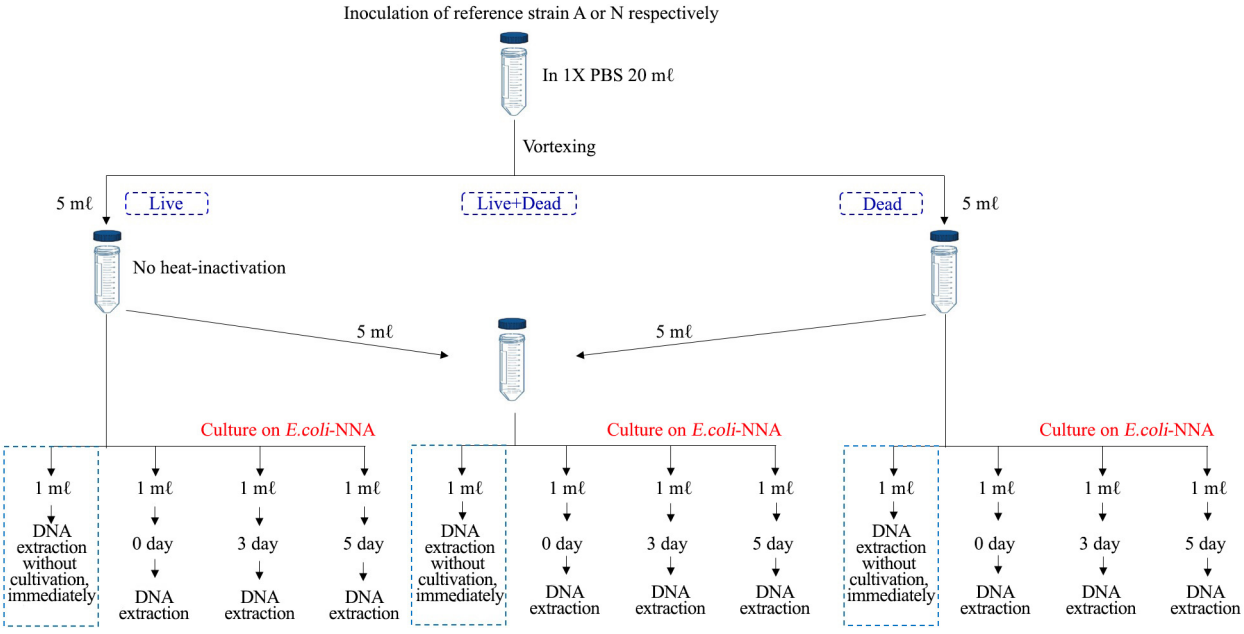

Fig. 2. Flow chart for experiment comparing the quantity of DNA obtained with and without preculturing of FLAs. A, *Acanthamoeba castellanii*; N, *Naegleria fowleri*; NNA, non-nutrient agar.

Table 1. Primers and probes used in real-time PCR for detecting *Acanthamoeba* spp. and *Naegleria fowleri*

Free-living amoebas	Primers and probes	Sequences	Size (bp)	Target genes	Accession number of reference sequences
Acanthamoeba spp.	Acant-F	CTTCTAAGGAAGGCAGCAG	142	18S rRNA	KF318462
	Acant-R	ACTTGCCCTCCAATTGTTAC			
	Acant-P	HEX-CGACACGGGGAGGTAGTGAC-BHQ1			
N. fowleri	N.fowl-F	GTGGTAGTAGTATTTGTGCTGAAAC	157	18S rRNA	KY062165
	N.fowl-R	GCAAACCTGAAAGGCCTTAT			
	N.fowl-P	FAM-CTGGGTATAGCAATATATTCAGGGGAGC-BHQ1			

5~7 days. The *E. coli* was inactivated by heating at 65°C for 30 min (Fig. 2).

Genomic DNA extraction and real-time PCR

Genomic DNA was extracted from the environmental samples and from the cultured reference strains as follows. Cultured FLAs were raked from the incubated plates using a scraper and were each suspended in 200 ml of phosphate buffered saline solution. The Qiagen DNA mini kit (Qiagen) was then used to extract the genomic DNA from each sample, according to the manufacturer's protocol. The extracted genomic DNA was then used as the template DNA for real-time PCR using our newly designed primers and probes (Table 1). The real-time PCR mixture (20 ml) was composed of 2X qPCR premix (10 ml), 4X oligo mix (5 ml), extracted genomic DNA from the water sample culture (2 ml), and nuclease-free water (3 ml). The real-time PCR conditions were as follows: one 15 min denaturation step at 95°C, followed by 45 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 45 sec. The real-time PCR was carried out using a Mic qPCR Cycler (Bio Molecular Systems).

Results and Discussion

Acanthamoeba spp. and *N. fowleri* are widespread in natural environments, including water. Since they can infect humans and cause fatal amoebic encephalitis, these FLAs are classified by the World Health Organization (WHO) as high health-impact

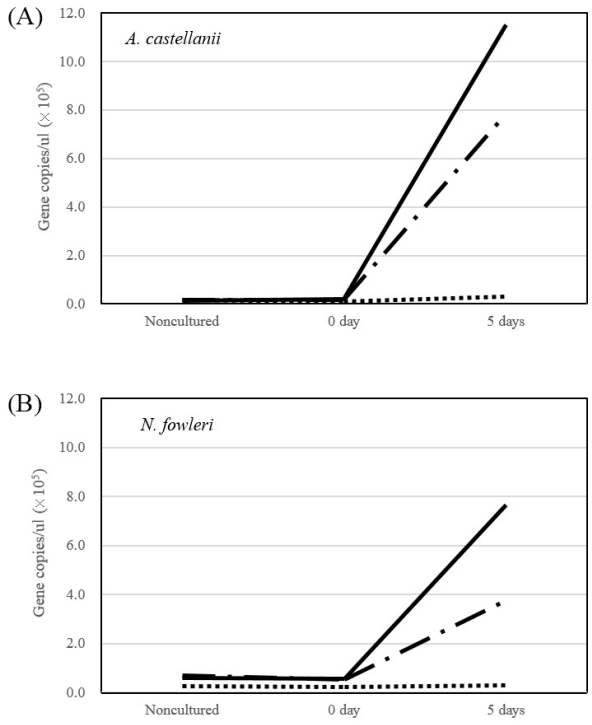

Fig. 3. Comparison of DNA amplification results for live, inactivated, and mixed amoebas after incubation. ——— live; — · — live + dead; ▪▪▪▪▪ dead.

pathogenic protozoa that can spread through drinking water. In the United States, they are on the CCL and are managed by the EPA. The WHO (2017) recommends specific monitoring of these amoebae when considering their distribution in the environment. Therefore, it is important to develop a reliable method to detect these FLAs in aquatic environments. Although most probable number (MPN) methods have been used to quantitatively detect FLAs in water environments, it has been reported that MPN methods might lead to underestimations (Thomas and Ashbolt, 2011). For this reason, various molecular biological techniques, such as PCR, nested PCR, and others, have been used in several studies (Marciano-Carbal and Cabral, 2003; Qvarnstrom *et al*., 2006; Derda *et al*., 2014). Among these techniques, real-time PCR has the advantage of high detection sensitivity and relatively low technical difficulty (Valasek and Repa, 2005). However, since molecular biological methods are based on the detection of nucleic acids, they have the disadvantage of being unable to determine whether the detected organisms are alive or dead. This is the most important disadvantage of applying molecular biological methods to detect microorganisms in environmental samples.

To compensate for this disadvantage, a method has been developed that can determine whether a target organism is present in selected environmental samples and whether the organism is alive. This method involves the completion of a pretreatment process using either propidium monoazide or ethylene methyl acrylate before nucleic acid extraction and PCR (Nam *et al*., 2011). In our present study, the concentrated water sample was incubated on non-nutrient agar with heat-inactivated *E. coli* as a food source for any amoebas before extracting genomic DNA from the sample and performing the real-time PCR. This method can indirectly determine whether culturable amoebas exist in the water samples, by comparing the results from nonculture-based real-time PCR with those from culture-based real-time PCR (Fig. 2).

To test this method, we first conducted experiments with heat-inactivated and live reference strains of *A. castellanii* and *N. fowleri*. In the inactivated amoeba samples, no increases in the quantity of genomic DNA were seen during incubation (Fig. 3). In contrast, in case of living amoebas, the quantity of DNA steadily increased as the samples were incubated. We then tested an inactivated amoeba sample mixed with a living amoeba suspension in a 1:1 ratio. This mixture showed a moderate increase in genomic DNA (Fig. 3). These results indicate that real-time PCR after an incubation stage serves not only as a way to detect FLAs more sensitively, but also as a way to indirectly determine whether the detected FLAs are culturable. This represents an important practical advance over conducting real-time PCR immediately after extracting the genomic DNA from concentrated water samples, a procedure that can only detect the presence of FLA DNA, which does not necessarily reflect the presence of viable FLAs.

There have been several reports of pathogenic FLAs in the Korean water system, albeit from peripheral and small-scale surveys (Jung *et al*., 2008; NIER, 2009; KCDC, 2014). The present study represents a larger survey using culture-based real-time PCR to search for viable FLAs in raw water that will be used for the source water of major water treatment plants in Korea. As this raw water will eventually become tap water and FLAs are able to survive during the water treatment process, it is important to estimate the presence. A total of 52 raw water samples were selected in 2017 (Fig. 1) and investigated for the

Table 2. Detection of *Acanthamoeba* spp. and *Naegleria fowleri* in water sources in Korea: season, source, and basin

		Number of samples	Number of positive samples				Prevalence of amoebas (%)			
			Acanthamoeba spp.		*N. fowleri*		*Acanthamoeba* spp.		*N. fowleri*	
			Noncultured	Cultured	Noncultured	Cultured	Noncultured	Cultured	Noncultured	Cultured
	Total	52	39	42	3	6	75.0	80.8	5.8	11.5
Season	Summer	20	12	16	0	3	60.0	80.0	0.0	15.0
	Autumn	30	25	24	3	3	83.3	80.0	10.0	10.0
	Winter	2	2	2	0	0	100.0	100.0	0.0	0.0
Source	Lake	40	31	32	3	5	77.5	80.0	7.5	16.1
	River	12	8	10	0	1	66.7	83.3	0.0	8.3
Basin	Han	9	5	7	1	2	55.6	77.8	11.1	22.2
	Geum/Seomjin	10	8	9	1	0	80.0	90.0	10.0	0.0
	Nakdong	20	14	16	0	3	70.0	80.0	0.0	15.0
	Yeongsan	2	2	2	0	0	100.0	100.0	0.0	0.0
	Others	11	10	8	1	1	90.9	72.7	9.1	9.1

presence of infectious *Acanthamoeba* spp. and *N. fowleri* using the culture-based real-time PCR assay. As a result, *Acanthamoeba* spp. were detected in 42 samples with a detection rate of 80.8%, and *N. fowleri* was detected in six samples with a detection rate of 11.5% (Table 2 and Supplementary data Table S1). Regarding differences between the water sources, the *Acanthamoeba* spp. showed no significant differences in detection rate between lake water (80.0%) and river water (83.3%), but the *N. fowleri* was detected slightly more often in lake water (16.1%) than river water (8.3%). *Acanthamoeba* spp. showed high detection rates (72.7~100%) in all water systems (Table 2). In contrast, *N. fowleri* was detected only in the Han River system (22.2%) and Nakdong River system (15.0%) with no detection in the Geum/Seomjin and Yeongsan River systems (Table 2).

Nonculture-based real-time PCR analysis detected *Acanthamoeba* spp. and *N. fowleri* in 39 (75.0%) and 3 (5.8%) samples, respectively (Table 2). These results indicated that culture-based real-time PCR is a more sensitive method for detecting FLAs from aquatic environmental samples than nonculture-based real-time PCR. *Acanthamoeba*, which has been very commonly detected in previous environmental studies (Marcino-Carbral and Carbral, 2003), was detected in 80.8% of the samples in the present study (Table 2).

N. fowleri is not as common as *Acanthamoeba* spp. (Visvesvara, 2010). In the present study, the detection rate of *N. fowleri* was also lower than that of *Acanthamoeba* spp. *N. fowleri* is an amoeba that prefers warm water, and has been detected at high rates during the hot summer season (Huizinga and McLaughlin, 1990; Hoffmann and Michel, 2001). We detected *N. fowleri* at high levels in the summer season, with 15.0% of the samples, and with high copy numbers for the detected DNA (Table 2 and Supplementary data Table S1).

N. fowleri was not detected in either of the two samples collected during winter (Table 2). In order to prevent infection by *N. fowleri*, it is necessary to intensively monitor and to properly control this amoeba during the summer season when water temperature rises. If this amoeba is detected as increased levels, it may be necessary for residents to refrain from some leisure activities such as swimming.

FLAs are strongly resistant to disinfectants such as chlorine (Thomas *et al*., 2004; Coulon *et al.*, 2010; Goudot *et al,* 2014). Infections through leisure activities in water such as swimming and tap water have also been reported (Shakoor *et al*., 2011; Yoder *et al*., 2012; Cope *et al.,* 2015). Therefore, it is necessary to completely remove the amoeba physically during the water treatment process by flocculation, sedimentation, and filtration.

The technique presented here can be used as a suitable method to discriminate whether amoebas detected in raw water or treated water are alive and monitor amoebas in treated water, tap water, and distribution systems, as well as raw water, in the future.

Conclusion

We developed a cell culture-based real-time PCR method for specifically detecting viable *Acanthamoeba* spp. and *N. fowleri*. Using this method, 52 samples from water sources were surveyed in 2017 for the presence of viable two FLAs. As a result, *Acanthamoeba* spp. were detected in 42 samples with a detection rate of 80.8%, and *N. fowleri* was detected in six samples with a detection rate of 11.5%. Nonculture-based real-time PCR analysis of these samples detected *Acanthamoeba* spp. and *N. fowleri* in 39 (75.0%) and 3 (5.8%) samples, respectively. These results indicate that real-time PCR after an incubation stage serves not only as a way to detect FLAs more sensitively, but also as a way to indirectly determine whether the detected FLAs are alive.

적 요

자유생활아메바인 가시아메바(*Acanthamoeba* spp.)와 파울러자유아메바(*Naegleria fowleri*)는 아메바성 뇌염 등 치명적인 질병을 일으키며, 물을 포함한 자연 환경에 널리 분포한다. 가시아메바와 파울러자유아메바가 한국의 주요 상수원수에 존재하는지 알아보기 위해 배양법에 기초한 실시간 중합효소연쇄반응법을 이용하여 2017년 7월부터 12월 사이에 한국의 52개 주요 상수원수를 조사하였다. 가시아메바와 파울러자유아메바가 각각 42개 시료(80.8%)와 6개 시료(11.5%)에서 검출되었다. 가시아메바의 경우 계절과 상관없이 고른 검출율을 보였으나, 파울러자유아메바는 주로 여름과 가을에 검출되었으며 겨울에는 검출되지 않았다. 이상의 결과는 이러한 자유생활아메바가 한국의 상수 원수에도 고루 존재한다는 것을 의미한다.

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