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Surveillance of *Acanthamoeba* spp. and *Naegleria fowleri* in environmental water by using the duplex real-time PCR

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Duplex real-time PCR을 이용한 수계 중 가시아메바와 파울러자유아메바 조사

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Naegleria fowleri and *Acanthamoeba* spp. are free-living amoebas that are widely distributed in natural environments. Although uncommon, infection with these protozoans can cause fatal disease in humans and animals. In this study, in order to select the appropriate method to survey *Naegleria fowleri* and *Acanthamoeba* spp. in water samples, four molecular biology techniques and one commercially available kit for real-time PCR were compared. The results indicated that the duplex real-time PCR was the most sensitive, and could be used to simultaneously detect two different free-living amoebas. Using the duplex real-time PCR approach, the two free-living amoebas were surveyed in three local streams in Daejeon, Republic of Korea. The concentrated free-living amoebas were inoculated onto non-nutrient agar plates which had been spread with heat-inactivated *Escherichia coli* and incubated for 5~7 days. After incubation, gDNA was extracted and used as the template for amplification by duplex real-time PCR. *Acanthamoeba* spp. and *N. fowleri* was detected from ten (83.3%) and two (16.6%) of the twelve samples, respectively. As these two free-living amoebas can be fatal, continuous surveillance is needed to track their distribution in the aquatic environment for the drinking water safety.

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Free-living amoebas (FLAs), also known as amphizoic amoebas, are largely distributed in natural environments, including water and soil, throughout the world (da Rocha-Azevedo *et al*., 2009). FLAs include four genera that infect humans or other animals: *Naegleria (N. fowleri* only), *Acanthamoeba*, *Balamuthia*, and *Sappinia* (Schuster and Visvesvara, 2004; da Rocha-Azevedo *et al*., 2009). Unlike general parasites, these pathogenic FLAs can freely survive in natural environments without using human or animal hosts.

N. fowleri, known popularly as the 'brain-eating amoeba', is a thermophilic FLA. It is commonly distributed in warm water (lakes, rivers, and hot springs) and soil but does not exist in seawater (De Jonckheere, 2012). *N. fowleri* is recognized as the sole species that causes infections in humans, among the more than 30 presently characterized species of *Naegleria* (De Jonckheere, 2004; Visvesvara *et al.*, 2007). Although *N. fowleri* is free-living in natural environments and survives through

predating bacteria, it may cause a fatal acute infection in a human host after traveling from the nose to the brain. This infection, known as primary amoebic meningoencephalitis, is contracted through human aquatic activities (Martinez and Visvesvara, 1997).

Acanthamoeba spp. are pathogenic in healthy people, causing serious amoebic keratitis (AK) in users of contact lenses (Marciano-Cabral and Cabral, 2003). Cases of keratitis caused by *Acanthamoeba* spp. have increased explosively in the past 20 years, in general due to increases in contact lens use and improper sanitary control of the lens (Seal and Hay, 1994; Awwad *et al*., 2007). In addition to AK, *Acanthamoeba* spp. may cause the opportunistic infection granulomatous amoebic encephalitis (GAE) in patients who have low immunity (Siddiqui and Khan, 2012). In Republic of Korea, two cases of *Acanthamoeba*-induced GAE have been seen. In both cases, the patients died because of brain inflammation (Im and Kim, 1998).

Therefore, the surveillance of these harmful *N. flowleri* and *Acanthamoeba* spp. in aquatic environments is important. Various molecular techniques such as PCR, nested PCR, loop-mediated isothermal amplification (LAMP), and real-time PCR have been developed for the detection of *N. floweri* (Marciano-Cabral *et al*., 2003; Qvarnstrom *et al*., 2006; Madarova *et al*., 2010; Mahittikorn *et al*., 2015) and *Acanthamoeba* spp. (Mathers *et al*., 2000; Schroeder *et al*., 2001; Yang *et al*., 2013; Derda *et al*., 2014). In addition, a real-time PCR kit for detecting *N. fowleri* and *Acanthamoeba* spp. is commercially available.

However, there have been few reports on the development of such detection methods and their use in surveillance of FLAs in the Republic of Korea. Jung *et al*. (2008) reported only the survey results for *Acanthamoeba* spp. in raw, settled, filtered, and treated water, according to the water treatment process in Busan metropolitan city.

Although it is important to investigate the presence of two FLAs in aquatic environments, there are no applicable or suitable national standard methods to survey two FLAs in Republic of Korea. Therefore, one commercially available and four previously described non-commercial molecular methods were compared in order to establish an optimal detection method for *Acanthamoeba* spp. and *N. fowleri* in environmental water samples. In addition, the surveillance of two FLAs was conducted for the first time in Korean aquatic environments.

Materials and Methods

Reference strains

Naegleria fowleri Carter (ATCC 30215) and *Acanthamoeba castellanii* (Douglas) Page (ATCC 30011) were purchased from the American Type Culture Collection. The *N. fowleri* and *A. castellanii* 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, in order to maintain active states.

Samples

Six sampling locations were selected from Gap stream, Yoodeung stream, and Daejeon stream, all of which are located in Daejeon, Republic of Korea (Fig. 1). At September and October in 2016, one liter of water was collected from each of six locations, in duplicate. The raw water samples, 970 ml each, were filtered using the polycarbonate filters (Isopore Membrane Filters, Merck Millipore Ltd.) with a pore size of 0.8 μm. The filter was submerged in 30 ml of the raw water sample and vortexed vigorously. Then, the filter was removed, and the

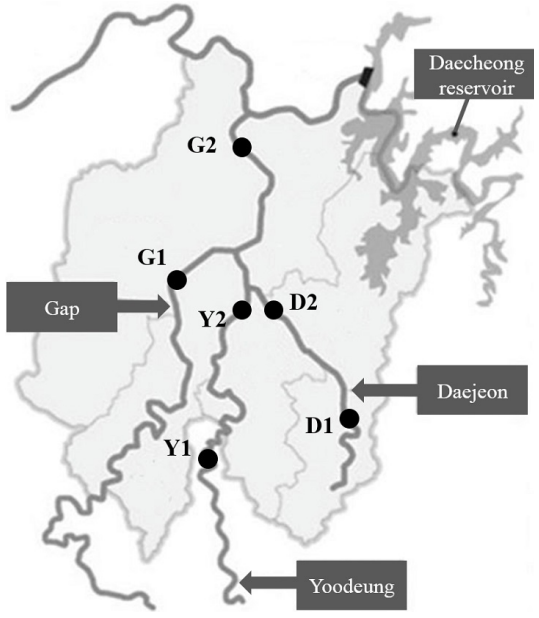

Fig. 1. Sampling locations of three streams in Daejeon city, Republic of Korea. GPS coordinates of D1, D2, G1, G2, Y1, and Y2 are 36°18'53.4"N 127°26'21.85"E, 36°26'25.71"N 127°3'17.24"E, 36°18'24.61"N 127°21'33.88"E, 36°21'30.38"N 127°21'30.38"E, 36°17'55.24"N 127°20'19.61"E, and 36°20'40.89"N 127°24'11.66"E, respectively.

resuspended mixture was centrifuged at 1,500 × *g* for 20 min. The supernatant was then removed, leaving a residue of 2 ml in the tube. The concentrated sample, 1 ml, was cultured on a non-nutrient agar (ATCC Medium 919) plate, prepared by pre-inoculating inactivated *Escherichia coli* (KCTC 2441, Korean Collection for Type Cultures) at 30°C for 5~7 days. The inactivated *E. coli* was prepared by heating at 65°C for 30 min.

Genomic DNA extraction

To extract the genomic DNA of FLAs, cultured FLAs were raked from the incubated plates using a scraper and were each suspended in phosphate buffered saline solution of 200 ml. Genomic DNA was then extracted from each sample using the Qiagen DNA mini kit (Qiagen) according to the manufacturer's protocol.

Molecular based techniques

In order to select the most appropriate method to detect *Acanthamoeba* spp. and *N. fowleri,* the detection sensitivity of four published molecular methods (PCR, real-time PCR, nested PCR, and LAMP) and one commercially available real-time PCR kit (Genesig) were compared. The primers used, as well as the references to the original papers describing the methods, are listed in Table 1. Each method was performed following the published or the manufacturer's protocol.

Results and Discussion

Comparison of the sensitivity of the molecular methods for detecting *N. fowleri* and *Acanthamoeba* spp. was conducted using ten-fold diluted (10^0~10^{-6}) solutions of genomic DNA (gDNA) extracted from *N. fowleri* and *A. castellanii*.

Table 1. The molecular methods for detecting *N. fowleri* and *Acanthamoeba* spp.

Free living amoeba	Methods	Primers or probes	Sequences	References
Acanthamoeba spp.	PCR	Aca 16S forward 1010	5'-TTATATTGACTTGTACAGGT GCT-3'	Derda *et al.* (2014)
		Aca 16S reverse 1180	5'-CATAATGATT TGACTTCTTCTCCT-3'	
	LAMP	F3	5'-GGCGACGATTCATTCAAAT-3'	Yang *et al.* (2013)
		B3	5'-CAAGACTCTTGTCGAGCGC-3'	
		FIP	5'-TCCCTCTCCGGAATCGAACCCTCGATGGTAGGATAGAGGCC-3'	
		BIP	5'-TTCTAAGGAAGGCAGCAGGCGTATTGTCACTACCTCCCCGT-3'	
		LF	5'-TCCGTTACCCGTTACGACCA-3'	
		LB	5'-CGCAAATTACCCAATCCCGAC-3'	
	Real-time PCR	AcantF900	5'-CCC AGATCGTTTACCGTGAA-3'	Qvarnstrom *et al.* (2006)
		AcantR1100	5'-TAAATATTAATG CCCCCAACTATCC-3'	
		AcantP1000	5'-HEX-CTGCCACCGAATACATTAGCATGG-BHQ3-3'	
N. fowleri	Nested PCR	Mp2Cl5 forward	5'-TCTAGAGATCCAACCAATGG-3'	Reveiller *et al.* (2003)
		Mp2Cl5 reverse	5'-ATTCTATTCACTCCACAATCC-3'	
		Mp2Cl5. for-in	5'-GTACATTGTTTTTATTAATTTCC-3'	
		Mp2Cl5. rev-in	5'-GTCTTTGTGAAAACATCACC-3'	
	LAMP	F3	5'-TGGATGGAGTAAGAGAGTTG-3'	Mahittikorn *et al.* (2015)
		B3	5'-TGAGTGTAGTTAATAATTCCTGTAC-3'	
		FIP	5'-GCAATGGATTGATTTGGAACGCAACAATGAAAGAAACTTTGCACCT-3'	
		BIP	5'-TTCCGTAGATTGGACGTCCATCCATCCATTTGGATCGG-3'	
	Real-time PCR	NaeglF192	5'-GTGCTGAAACCTAGCTATTGTAACTCAGT-3'	Qvarnstrom *et al.* (2006)
		NaeglR344	5'-CACTAGAAAAAGCAAACCTGAAAGG-3'	
		NfowlF	5'-FAM-ATAGCAATATATTCAGGGGAGCTGGGC-BHQ1-3'	

The gDNAs of *N. fowleri* and *A. castellanii* were detected in 10^0~10^{-5} diluted samples by using the commercial real-time PCR kit (Table 2). The copy numbers of the diluted *N. fowleri* gDNA for the 10^0~10^{-5} dilutions were 7.7×10^6 copies/ml, 7.7×10^5 copies /ml, 8.2×10^4 copies/ml, 8.8×10^3 copies/ml, 1.0×10^3 copies/ml, and 171 copies/ml. The copy numbers of the diluted *A. castellanii* gDNA for the 10^0~10^{-5} dilutions were 2.4×10^6 copies/ml, 4.6×10^4 copies/ml, 4.0×10^3 copies/ml, 334 copies/ml, 25 copies/ml, and 3 copies/ml. No gDNA was detected at dilutions of 10^{-6}.

The duplex real-time PCR was conducted to detect gDNA of *N. fowleri* and *A. castellanii*. The results showed that the gDNA of *N. fowleri* and *A. castellanii* was detected in 10^0~10^{-5} diluted samples by duplex real-time PCR (Table 2).

Nested PCR was used to amplify gDNA from the type strain of *N. fowleri.* The nested PCR yielded clear bands for dilutions of 10^0~10^{-3}. However, it was not possible to verify any bands for dilutions of 10^{-4}~10^{-6} (Table 2). PCR was done for *A. castellanii* gDNA, using Aca 16S primers. As seen with *N. fowleri*, the amplification resulted in clear bands for dilutions of 10^0~10^{-3}, and there were no bands for dilutions of 10^{-4}~10^{-6} (Table 2).

LAMP analysis was conducted using one pair of outer primers, one pair of inner primers, and one pair of loop primers. The results showed the typical ladder shapes for the LAMP products amplified from dilutions of 10^0~10^{-3}. No bands were seen for dilutions of 10^{-4}~10^{-6}. In the case of *A. castellanii*, as with *N. fowleri*, the results showed typical ladder shapes for the LAMP products amplified from dilutions of 10^0~10^{-3}, and there were no ladder shapes for dilutions of 10^{-4}~10^{-6} (Table 2).

The results showed that the duplex real-time PCR and the commercial simplex real-time PCR were highly sensitive among

Table 2. Comparison of the three different molecular methods in terms of detection sensitivity

Detection methods	Amoeba	10-fold dilution						
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Real-time PCR	*A. castellanii*	+	+	+	+	+	+	-
	N. fowleri	+	+	+	+	+	+	-
Duplex real-time PCR	*A. castellanii*	+	+	+	+	+	+	-
	N. fowleri	+	+	+	+	+	+	-
PCR[a]	*A. castellanii*	+	+	+	+	-	-	-
	N. fowleri	+	+	+	+	-	-	-
LAMP	*A. castellanii*	+	+	+	+	-	-	-
	N. fowleri	+	+	+	+	-	-	-

[a]*A. castellanii*, PCR; *N. fowleri*, nested PCR

Table 3. Results of the raw water analysis in three streams in Daejeon

Stream	Site	Month	pH	Temp.	Turbidity (NTU)	*N. fowleri*	*Acanthamoeba* spp.
Daejeon	D1	Sep.	8.7	28.6	1.25	-	+
		Oct.	7.8	16.5	0.93	+	+
	D2	Sep.	8.6	27.6	2.68	-	-
		Oct.	7.0	18.1	5.81	-	+
Gap	G1	Sep.	7.9	26.4	0.96	-	-
		Oct.	7.8	15.1	1.56	-	+
	G2	Sep.	7.3	26.2	2.08	-	+
		Oct.	8.0	18.4	5.16	-	+
Yoodeung	Y1	Sep.	8.5	27.4	1.02	-	+
		Oct.	8.1	16.4	2.56	-	+
	Y2	Sep.	8.3	28.5	1.73	-	+
		Oct.	8.2	16.0	2.92	+	+

the compared molecular methods and the sensitivity of two methods was similar. However, we used the duplex real-time PCR for the surveillance of the FLAs in the aquatic environment of Daejeon city as the duplex real-time PCR could detect the two FLAs simultaneously in the single tube. In Table 3, *Acanthamoeba* spp. were detected in all samples except only two samples from the D2 and G1 sites, collected at September 2016. *N. fowleri* was detected in the samples from the D1 and Y2, collected at October 2016. Albeit the number of the surveyed sites and the samples was small, we firstly showed that the two harmful FLAs are distributed in the aquatic environment of the Republic of Korea.

According to De Jonckheere (2012), *N. fowleri* has been found on all continents except Antarctica. Also, it is known that *Acanthamoeba* spp. are also found throughout the world (da Rocha-Azevedo *et al*., 2009). They are classified as pathogenic protozoa in the contaminants candidate list of the U.S. Environmental Protection Agency. They are generally considered as organisms that need to be monitored and controlled to ensure drinking water safety, even though no legal regulations regarding amoebas specifically exist in a number of countries including Canada and Australia. In the Republic of Korea, there have been few environmental science studies on *N. fowleri* and *Acanthamoeba* spp., other than a few clinical studies (Jeong and Yu, 2005; Kim *et al*, 2008; Lee *et al*., 2011; Moon *et al*, 2016).

The high sterilization resistance of these FLAs becomes a risk factor in the drinking water treatment process. De Jonckheere *et al*. (1976) and Sarkar and Gerba (2012) reported that *Acanthamoeba* spp., were highly resistant to chlorine and UV light, and UV resistance was even higher than that of *Cryptosporidium* oocysts. De Jonckheere *et al*. (1976) found that *N. fowleri* showed high sterilization resistance. Cursons *et al*. (1980) reported that the inactivation of cysts required exposure to a residual chorine concentration of 0.74 mg/L for 30 min. Thus, it is necessary to investigate the presence and distribution of these FLAs in environmental water for the safety of drinking water. In addition to high sterilization resistance, these amoebas host other microorganisms such as bacteria, mold, and viruses and they can release these microorganisms into environments (Greub and Raoult, 2004; Thomas *et al*., 2004, 2010; Richards *et al*., 2013; Denoncourt *et al*., 2014). Also, it has been recognized that bacteria inside the amoebas can show high resistance to various stresses and sanitizers. This is another impetus for investigating the ecology of amoebas; such knowledge is necessary to determine the nature and prevalence of dangerous microbiological factors in our drinking water and water sources and to develop water management plans.

Conclusion

We verified that real-time PCR was highly sensitive compared to PCR and LAMP as detection methods for two FLAs. Further, the duplex real-time PCR assay method could detect the two FLAs simultaneously. Among the twelve samples in three streams located in Daejeon city, *N. fowleri* and *Acanthamoeba* spp. were detected in two (16.6%) and ten (83.3%) samples, respectively. Although we tested only 12 samples, the results showed that the harmful FLAs were distributed in the Korean aquatic environment and *Acanthamoeba* was detected more frequently than *N. fowleri*. This implies that water resources should be managed and well treated to ensure the safety of public drinking water. In addition, it is necessary to survey the distributions of the two FLAs continuously in nationwide for maintaining the drinking water safety in Republic of Korea.

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적 요

가시아메바(*Acanthamoeba* spp.)와 파울러자유아메바(*Naegleria fowleri*)는 자유생활아메바로 자연계에 널리 분포하며 사람과 동물에게 치명적인 질병을 일으킨다. 본 연구에서는 가시아메바와 파울러자유아메바를 물 환경에서 조사하기 위해 기존에 보고된 네 종류의 분자생물학적 방법과 상용 real-time PCR 키트의 분석 민감도를 비교하였다. 그 결과 duplex real-time PCR 방법이 민감도가 가장 좋았으며, 동시에 두 종류의 자유생활아메바를 검출할 수 있었다. 따라서 이 방법을 사용하여 한국의 대전시에 위치한 3개 하천, 6개 지점을

대상으로 그 분포를 2회 조사하였다. 가시아메바는 12개 시료 중 10개 시료에서 검출되었으며(83.3%), 파울러자유아메바는 2개 시료에서 검출되었다(16.6%). 향후 이러한 유해 아메바로부터 먹는 물의 안전성을 확보하기 위해 지속적인 분포 조사가 필요할 것이다.

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