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Short Communication

Isolation and Identification of *Naegleria* Species from Environmental Water in Changchun, Northeastern China

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Abstract

Background: *Naegleria* is a free-living amoeba, and pathogenic *Naegleria* may pose a health risk to people exposed to recreational water. Our objective in this study was to determine if there are pathogenic amoebae in environmental water samples from Changchun, Northeastern China.

Methods: During July to September 2012, a total of 70 water samples were collected from Changchun, Northeastern China, and *Naegleria* was enriched by in vitro culture and detected by PCR using *Naegleria* genus-specific primers. Resulting PCR products were sequenced and phylogenetically analyzed to identify *Naegleria* species.

Results: *Naegleria* was detected in 65 (92.9%) of 70 water samples. DNA sequence and phylogenetic analyses based on the internal transcribed spacer (ITS) rDNA sequences revealed four *Naegleria* species, including *N. pagei* (n = 24) and *N. Australiensis* (n = 18), *N. clarki* (n = 13) and *N. gruberi* (n = 10), in which *N. australiensis* is pathogenic to mice. But the pathogenic species *N. fowleri* was not detected.

Conclusion: This is the first report on *Naegleria* species in Northeastern China, showing that almost all environmental water samples were contaminated with *Naegleria*, including *N. pagei*, *N. Australiensis*, *N. clarki* and *N. gruberi*, which should be considered a potential public health threat.

Introduction

Free-living amoebae (FLA), feeding on bacteria, fungi, and algae, are ubiquitous in soil and aquatic environments. Of the hundreds of FLA species, *Naegleria* is one of the predominant FLA found in lakes, rivers, swimming pools, hot spring, geother-

mal water, discharge from industrial plants (1). More than 30 species of the genus *Naegleria*, belonging to the family *Vahlkampfiidae*, have been identified using molecular techniques, in which *N. fowleri* is the only species pathogenic to humans (2). *N. fowleri* is transmitted via the nasal mucosa and the olfactory nerve to the brain, causing primary amoebic meningoencephalitis (PAM), an acute and rapidly fatal disease of people following exposure to polluted water (3).

The human case of PAM was first described in Australia in 1965, subsequently found in Florida and Texas in the United States (US). A retrospective study has indicated that the earliest case occurred in Richmond, Virginia from 1951 to 1952 (4). So far approximately 300 cases have been documented worldwide, mostly in USA, Australia and Europe (5, 6). These diseases are almost uniformly fatal with only several survivors. In Asia, the disease has been found in Thailand (7, 8), India (9), Iran (10), and Pakistan (11). In Pakistan, at least 10 people died of the disease in 2009 (12). In China, only 5 cases had been reported in Henan, Hebei and Hainan provinces, respectively, before 2005 (13). However, some cases of PAM may be unreported, as it is difficult to diagnose.

Our objective in this study was to determine if there are pathogenic amoebae in environmental water samples from Changchun, Northeastern China by microbial culture combined with molecular methods.

Materials and Methods

Sample collection

The water samples were collected from Changchun Park (CCP), Zhaoyang Park (ZP), Jingyue Park (JP), Nanhu Park (NP), Yitong River (YR), Xinlicheng River (XR), Children Park (CDP) in Changchun City (125.35°E; 43.88°N), Jilin Province, Northeastern China. The water samples (2 L) were collected within 10 cm of the water surface using a sterile polypropylene bottle and transported to the laboratory for subsequent analyses.

Naegleria detection

To investigate *Naegleria* in environmental water body, water samples were filtered through 45-mm diameter cellulose nitrate membranes (Pall, USA) with a pore size of 5 μm (14). The water samples were filtered through three to six cellulose nitrate membranes, which were subsequently inverted and placed onto 1.5% non-nutrient agar (NNA) plates containing a lawn of *Escherichia coli* (15, 16). 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 37°C for 14 days.

The positive *Naegleria*-like samples were detected by PCR using *Naegleria* genus-specific primers (5'-GAACCTGCGTAGGGATCATTT-3' and 5'-TTTCTTTTCCTCCCCTTATTA-3'), and *Naegleria fowleri* species-specific primers (5'-GTGAAAACCTTTTTTTCCATTTACA-3' and 5'-AAATAAAAGATTGACCATTTGAAA-3') (17, 18). PCR reactions were performed as described as elsewhere (19). Then PCR products were separated on a 1% agarose gel. Products were visualized by ethidium bromide staining and imaged under UV light.

Sequencing and phylogenetic analysis

Resulting PCR products were sequenced and compared with each other and with reference sequences. Phylogenetic analyses based on the ITS and 5.8S sequences were conducted using the software MEGA 4 (http://www.megasoftware.net/) (20). The reliability of branches in the tree was assessed by bootstrap analysis with 1,000 replicates.

For comparative phylogenetic analysis, the following sequences were retrieved from the GenBank: *N. americana* (AJ566623), *N. andersoni* (X96572), *N. australiensis* (AY293307), *N. canariensis* (AJ973124), *N. clarki* (GU597045), *N. fowleri* (X96567), *N. galeacystis* (X96578), *N. gruberi* (AJ132031), *N. indonesiensis* (AJ243444), *N. italica* (X96574), *N. jamiesoni* (X96570), *N.*

laresi (AJ566630), *N. lovaniensis* (X96568), *N. minor* (X96577), *N. pagei* (AJ566633), *N. peruana* (AJ785757), *N. philippinensis* (AM167890), *N. pussardi* (X96571), *N. tihangensis* (AJ566631).

Results

A total of 70 environmental water samples were collected from CCP, ZP, JP, NP, YR, XR, and CDP in Changchun, Northern China, 10 samples in each site (Table 1). The water samples were filtered through cellulose nitrate membranes, and cultured on NNA plates with *Escherichia coli* at 37°C. *Naegleria*-like trophozoites were normally observed after 48 or 72 hours of culture, and the cyst stage could be seen after at least three days. The flagellate stage could not usually be seen unless induced by sterile distilled water to the surface agar. After 14 days of culture, 65 water samples (92.9%) were found positive for *Naegleria*-like trophozoites.The 65 isolates were further identified by PCR using *N. fowleri* species-specific primers and *Naegleria* genus-specific primers.

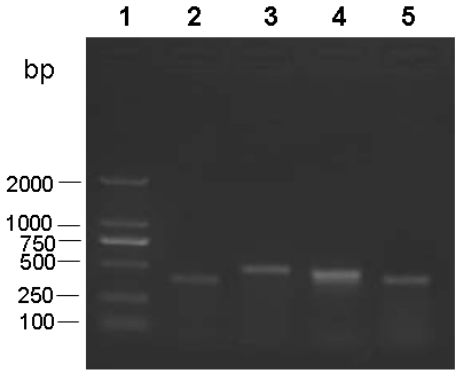

Fig. 1: Amplicon bands revealed by genus-specific primers against *Naegleria*-like isolates. Lane 1 represents the 2,000-bp DNA ladder (Takara); Lanes 2–5 represent the amplicon bands with 379-bp, 495-bp, 460-bp and 395-bp against the different water samples using the *Naegleria* genus-specific primers, which were grouped into *N. gruberi*, *N. clarki*, *N. pagei,* and *N. Australiensis*, respectively

The *N. fowleri* species-specific primers did not show any amplicon bands against all 65 isolates. In contrast, *Naegleria* genus-specific primers produced amplicons against all the isolates, which could be differentiated into 4 groups represented by 379-bp, 495-bp, 460-bp and 395-bp, respectively (Fig. 1). Resulting PCR products were sequenced and compared with each other and with reference sequences. The unique sequences have been submitted to the GenBank database (JX413059, JX267141, JX294421, and JX861079). DNA sequence showed the presence of four *Naegleria* species in Changchun, including *N. gruberi*, *N. clarki*, *N. pagei,* and *N. Australiensis* (Fig. 2). Among the 65 positive samples, 24 (34.3%) were *N. pagei,* which were the dominant species; 18 (25.7%) were *N. Australiensis;* 13 (18.6%) were *N. clarki*; and 10 (14.3%) were *N. gruberi.*

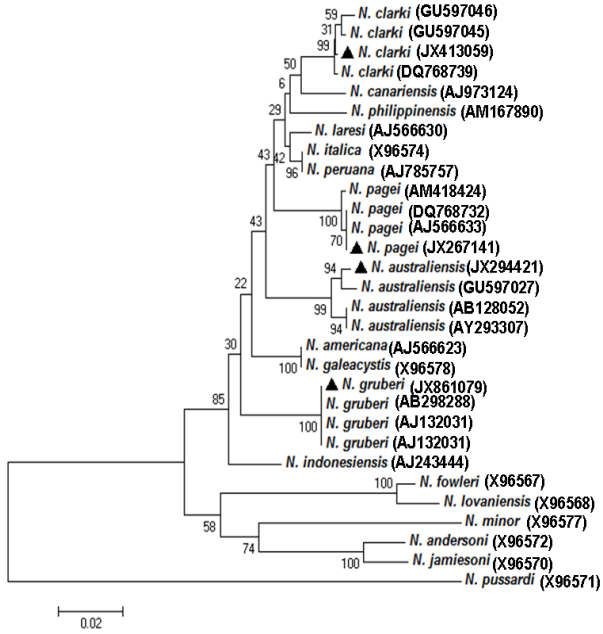

Fig. 2: Phylogenetic relationships of *Naegleria* identified in this study. A neighbor-joining analysis of the internal transcribed spacer (ITS) rDNA sequences was conducted using the software MEGA 4. Numbers on branches are percent bootstrapping values (>50) using 1,000 replicates. The triangles represent the *Naegleria* species in the present study

Table 1: *Naegleria* species in environmental water samples from Changchun, Northeastern China

Sampling site	No. of sample	*Naegleria* species (%)			
		N. australiensis	***N. pagei***	***N. clarki***	***N. gruberi***
Changchun Park (CCP)	10	4 (40.0)	5 (50.0)	0	0
Zhaoyang Park (ZP)	10	0	10 (100.0)	0	0
Jingyue Park (JP)	10	1 (10.0)	3 (30.0)	6 (60.0)	0
Nanhu Park (NP)	10	4 (40.0)	3 (30.0)	0	0
Yitong River (YR)	10	9 (90.0)	0	0	0
Xinlicheng Reservoir (XR)	10	0	3 (30.0)	7 (70.0)	0
Children Park (CDP)	10	0	0	0	10 (100.0)
Total	70	18 (25.7)	24 (34.3)	13 (18.6)	10 (14.3)

Discussion

In this study, four *Naegleria* species, including *N. gruberi*, *N. clarki*, *N. pagei,* and *N. Australiensis,* were detected from 65 (92.9%) water samples in Changchun, Northeastern China. Based on the sequence of the internal transcribed spacer (ITS) rDNA sequences, the *N. gruberi* isolate was most similar (99%) to *N. gruberi* (AB298288) in Japan, the *N. clarki* and *N. australiensis* isolates were 99% similar to *N. clarki* (GU597046) and *N. australiensis* (GU597027) in Taiwan, and the *N. pagei* isolate showed 100% identity to *N. pagei* (AJ566633) in USA. Phylogenetic analysis demonstrated that the four *Naegleria* species formed a clade with *N. gruberi*, *N. clarki*, *N. pagei,* and *N. Australiensis,* respectively, and the bootstrap values for their inclusions were high (Fig. 2).

The most pathogenic *Naegleria* species, *N. fowleri*, was not identified in the detected water samples from Changchun, China. This may explain why PAME cases have not been reported in the studied areas. The *Naegleria* species found in the present study are also identified in other countries and regions. For example, *N. australiensis* is distributed in Europe, North America, and Australia. *N. pagei* is present in Europe, North America, Africa, and Asia. *N. clarki* is found in Australia, Africa, *N. gruberi* is found in Europe, Asia, North America (21).

N. australiensis has been shown to be responsible for the majority of pathogenic isolates in Oklahoma, which can also found in fish brains (22, 23). Mice inoculated intranasally with *N. australiensis* at 1×10^5 cell exhibits an average mortality rate of 20% (24). *N. australiensis* is regarded as a potentially pathogenic species by ATCC, although it has never been found in a human (25). However, *N. australiensis* has been identified in fish brains.

N. pagei, *N. clarki*, and *N. gruberi* were non-pathogenic. However, some studies report that *Legionella pneumophila*, which causes Legionnaire's disease, can replicate in amoeba host, leading to infection and subsequent intracellular replication (26, 27). Thus, *N. gruberi* hosting *L. pneumophila* in aquatic environments may have serious health consequences. *N. clarki* can also act as vehicle for bacteria, which can harbor two different bacterial populations in the cytoplasm and in the nucleus, respectively (28). In addition, it has demonstrated that the pathogenic *N. fowleri* is evolved from the non-pathogenic *N. lovaniensis* (6).

Conclusion

This is the first report on *Naegleria* species in Northeastern China, showing that almost all environmental water samples were contaminated with *Naegleria*, including *N. pagei*, *N. Australiensis*, *N. clarki* and *N. gruberi*, which should be considered a potential public health

threat, as *N. australiensis* is pathogenic to mice, and *N. gruberi* may host *L. pneumophila*.

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