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Detection of *Naegleria* sp. in a Thermal, Acidic Stream in Yellowstone National Park

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ABSTRACT. An initial survey of sequences of PCR-amplified portions of the 18S rRNA genes from a community DNA clone library, prepared from an algal mat in a thermal, acidic stream in Yellowstone National Park, WY, USA, revealed among other sequences, several that matched *Vahlkampfia*. This finding prompted further investigation using primers specific for *Naegleria*. Sequences from a subsequent DNA clone library, prepared from the 5.8S rRNA gene and the adjacent internal transcribed spacer (ITS) regions of the rRNA, closely matched *Naegleria* and formed an independent lineage within a clade containing *Naegleria sturti* and *Naegleria niuginiensis*. The sequences may represent a new *Naegleria* species.

Key Words. Amoebic meningoencephalitis, phylogenetics, ribosomal RNA, Vahlkampfiidae.

FREE-living vahlkampfiid amoebae occur worldwide, inhabiting soil and aquatic environments. A few species are thermophilic and able to thrive in naturally and artificially heated water such as lakes, spas, and hot springs where they live as opportunistic phagotrophs (De Jonckheere 2002). Given the right conditions, these amoebae can infect humans and other mammals. One vahlkampfiid, *Naegleria fowleri*, causes primary amoebic meningoencephalitis (John 1998), a rapidly fatal disease that usually occurs in persons with a history of swimming in warm water (Martinez and Visvesvara 1997). Because of the potentially fatal consequences of infection by *N. fowleri*, a reliable and rapid means of detecting these organisms in environmental samples is important. Since morphological differences are few, microscopic identification is impossible. Culturing methods enable the detection of only those organisms amenable to growth under defined conditions. Sequence analyses of cloned rRNA genes and internal transcribed spacer regions (ITS1 and ITS2), PCR-amplified directly from Nymph Creek, a thermal, acidic stream in Yellowstone National Park (YNP), WY, USA, provided a rapid means of detecting vahlkampfiid amoebae (De Jonckheere 2002; Pélandakis et al. 2000). Our results suggested the presence of a new, potentially pathogenic *Naegleria* species.

MATERIALS AND METHODS

Site description. Nymph Creek is a thermal (55 °C at its source), acidic (pH 2.7) spring-fed stream that flows for about 150 m to Nymph Lake. Its most striking feature is a vivid green algal mat that varies from a thin film (~ 1 mm) near its source to a more typical 1–2 cm thick mat at cooler temperatures downstream. The mat is composed predominately of red and green algae (Brock 1978; Doemel and Brock 1971; MJF, unpubl.).

Environmental parameters. Stream temperature was monitored at the collection site using HOBO XT data loggers (Onset Computer Corporation, Bourne, MA). Water samples were collected seasonally and analyzed for chemical elements by the Montana State University Soil Analysis Laboratory using an ICP emission spectrophotometer.

Nucleic acid extraction. Mat samples were collected on 4 April 2000 and 7 December 2000. DNA was extracted using a modification of the Moré et al. (1994) method as follows: a pea-sized sample of mat was bead-beaten in 800 μl of 120 mM $NaPO_4$ buffer (pH 8) and 260 μl of SDS solution (10% SDS, 0.5 M Tris-HCl pH 8, 0.1 M NaCl) using zirconium beads (0.1-mm diam., Biospec Products, Bartlesville, OK) for 45 s at speed 6.5 in a Savant Fast Prep bead beater (Savant Instruments Inc., Farmingdale, NY). The upper layer was removed, centrifuged for 3 min at 16,000 *g*, precipitated with 0.4 vol. of 10 M ammonium acetate for 5 min on ice, and then centrifuged for 5 min at 16,000 *g*. The supernatant fluid was precipitated with 0.7 vol. of isopropanol for 5 min at room temperature, followed by centrifugation at 16,000 *g* for 30 min at 4 °C. The pellet was rinsed twice with 70% ethanol, centrifuged for 5 min at 16,000 *g*, dried, and resuspended in 30 μl of molecular-grade water (Sigma, St. Louis, MO).

PCR amplification, cloning, sequencing, and phylogenetic analysis. Microbial populations were surveyed using PCR amplification, cloning, and sequencing approaches to detect taxonomically informative 18S rRNA gene sequences directly from DNA extracted from the mat. PCR primer sequences were 5′-ACCTGGTTGATCCTGCCA-3′ and 5′-TGATCCTTCYGCAGGTTCAC-3′, complementary to regions of conserved sequences proximal to 5′ and 3′ termini of the 18S rRNA gene as described by Moon-Van der Staay et al. (2001). PCR amplification was performed according to the manufacturer's instructions using a *Taq* DNA polymerase kit and dNTP mix (Promega Corporation, Fitchburg, WI). PCR conditions were 94 °C for 2 min followed by 30 cycles of 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 2 min. PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide. Individual bands of the predicted size were purified with a Bio101 gel extraction kit (Bio 101, Inc., Carlsbad, CA).

PCR-amplification also was performed with a second primer set 5′-GAACCTGCGTAGGGATCATTT-3′ and 5′-TTTCTTTTCCTCCCCTTATTA-3′ that amplifies the complete 5.8S RNA gene and the internal transcribed spacer regions (ITS1 and ITS2) as described by Pélandakis et al. (2000). Reaction conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 15 s, 55 °C for 1 min 30 s, 72 °C for 1 min 30 s, and extension at 72 °C for 10 min.

An additional PCR amplification was performed with an *N. fowleri*-specific primer set (Fw1 and Fw2) that complements the ITS-1 and ITS-2 regions (Pélandakis et al. 2000) according to the published method. All primers were obtained from IDT DNA, Coralville, IA.

PCR products were cloned using an Invitrogen TA cloning kit (Invitrogen, Carlsbad, CA) to produce clone libraries (Vergin et al. 2001). Individual colonies were selected, plasmid DNA was isolated using the Promega Plus SV miniprep DNA purification system (Promega Corporation, Fitchburg, WI) and assayed for the presence of an insert. Cycle sequencing of plasmid inserts was performed using AmpliTaq DNA Polymerase, M13 forward and reverse sequencing primers, FS, and Big Dye Terminators (Applied Biosystems, Foster City, CA). Sequences

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Table **1**. Strains used for phylogenetic analyses, their geographical origins, and GenBank database accession numbers.

Species	Origin	Accession number
Naegleria andersoni	Australia	X96572
Naegleria jamiesoni	Singapore	X96570
Naegleria carteri	Australia	Y10197
Naegleria galeacystis	USA	X96578
Naegleria gruberi	Australia	AJ132032
Naegleria italica	Italy	X96574
Naegleria fowleri	France	AJ132019
Naegleria fowleri	France	AJ132028
Naegleria morganensis	Australia	Y10192
Naegleria lovaniensis	France	X96569
Naegleria lovaniensis	France	X96568
Naegleria lovaniensis	Australia	Y10191
Naegleria pussardi	France	X96571
Naegleria sturti	Australia	Y10195
Naegleria niuginiensis	New Guinea	Y10193
YNP sequence (18S)	USA	*AY268958*
YNP sequence (ITS)	USA	*A4267537*

were aligned and manually edited using Sequencher 3.1.1 software (Gene Codes Corporation, Ann Arbor, MI) and compared to sequences in GenBank (Benson et al. 1999) using BLAST (Altschul et al. 1997). Phylogenetic analyses were performed using the Neighbor-joining algorithm (Saitou and Nei 1997) within PAUP* 4.0b8 (Swofford 2001) with distance correction set to the Kimura two-parameter model (Kimura 1980). GenBank/EMBL accession numbers and origins for the sequences used in the phylogenetic tree are listed in Table 1.

RESULTS AND DISCUSSION

The 18S rRNA primers were chosen for a survey of eukaryotic microbial populations in Nymph Creek. Among the clones analyzed, partial sequence analyses (416 bp) of four 18S rRNA gene clones showed > 99% sequence identity to each other. A BLAST search analysis of these sequences indicated that they were most similar (95%) to the 18S rRNA gene of *Vahlkampfia lobospinosa* (M98052). We did not identify any *Naegleria* clones in this library. Our failure to detect *Naegleria* could be due to primer bias caused by mixed template annealing in the amplification of the community DNA (Suzuki and Giovannoni 1996). The presence of a vahlkampfiid sequence in the initial clone library prompted us to test more specifically for the presence of *Naegleria* species. We examined environmental clones PCR-amplified with a primer set that brackets the more highly variable ITS region of the rRNA operon, used to differentiate organisms within this genus (De Jonckheere 1998; De Jonckheere and Brown 1997; Kilvington and Beeching 1995; Pélandakis et al. 2000). Twelve clone sequences from the ITS clone library showed > 99% identity to each other and 95% similarity to *N. sturti* (Y10195) in a BLAST search analysis.

To evaluate the taxonomic relationship between the YNP clone sequence and other *Naegleria* species, we constructed a phylogenetic tree using the 5.8S rRNA gene and ITS1 and ITS2 regions from a broad range of widely distributed species of major lineages of *Naegleria* (Fig. 1), described and sequenced by Pélandakis et al. (2000; Fig. 3) and De Jonckheere (2002). The YNP clone sequence formed an independent lineage within a clade containing *N. sturti* and *N. niuginiensis*. However, support for the inclusion of the YNP clone sequence within this clade was low (bootstrap value = 52%). The genetic distance between the YNP clone sequence and its nearest relative was at least as great as that separating other *Naegleria* species in

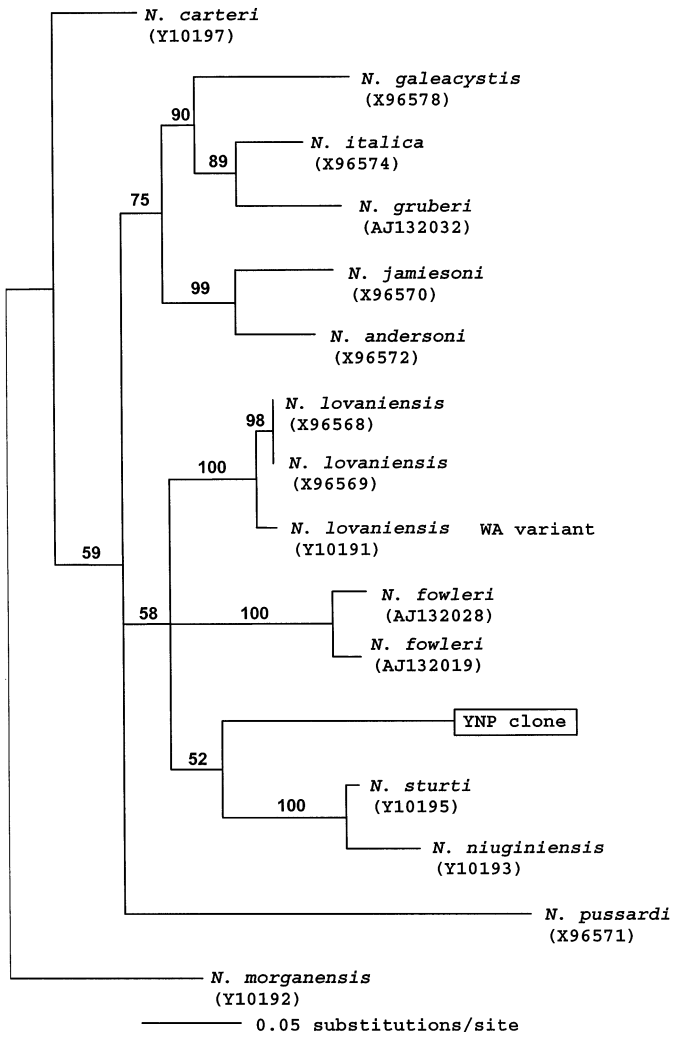

Fig. **1**. A Neighbor-joining tree based upon a comparative analysis of a 327-bp ITS rDNA sequence from YNP with other GenBank *Naegleria* sequences. The tree was generated using PAUP* and distances were calculated by the Kimura two-parameter model. Bootstrap values > 50% are given.

the phylogenetic analysis, suggesting that the YNP clone sequence represented a new species.

Alignments with *Naegleria* sequences in GenBank showed that the length of the YNP clone ITS1, 5.8S rRNA gene, and ITS2 sequences are 35, 175, and 117 bp, respectively. Compared with values reported by De Jonckheere (Table 2, 2002), the YNP clone sequence is most similar to *N. sturti* (Y10195), varying in length by only 1 bp in the ITS2.

An *N. fowleri* species-specific primer set (Pélandakis et al. 2000) failed to yield a PCR product when used to amplify Nymph Creek DNA suggesting that the YNP organism is genetically distinct from those of *Naegleria fowleri* isolates available in the database.

Using cultivation-based detection methods, Ramaley and colleagues sampled acidic, neutral, and alkaline environments in YNP and Grand Teton National Park. They successfully isolated *Naegleria* sp. from neutral to slightly alkaline springs but were unable to obtain isolates from acidic springs (Ramaley et al. 2001). Using light microscopy, we established the presence of vahlkampfiids in Nymph Creek (data not shown). Because genera and species cannot be identified by light microscopy

alone and because previous studies by Ramaley and colleagues (Ramaley et al. 2001) suggested that *Naegleria* sp. are not readily cultivated from acidic hot springs in YNP, we used molecular analyses to identify amoebae in situ. We detected amoebae seasonally and in multiple samples.

We monitored environmental parameters at Nymph Creek in order to relate stream conditions to the organisms present. Temperatures ranged from 41.2 °C to 52.6 °C from March 2000 to December 2000 (data not shown) and were within the growth range for thermophilic *Naegleria* sp. (De Jonckheere and Van de Voorde 1977). Water chemistry data showed elevated levels of iron (2.85 mg/L), sulfur (97.27 mg/L), arsenic (0.113 mg/L), and manganese (0.11 mg/L). These values were stable seasonally, and are characteristic of acidic environments in geothermal areas (Brock 1978). The pH was 2.7 throughout our study and remarkably, is consistent with values reported over 20 years ago by Brock (Brock 1978).

We suggest that *Naegleria* thrive in Nymph Creek, are not transient entities, and that the sequence-type detected may represent a new, potentially pathogenic *Naegleria* species adapted to a high temperature, low pH, metal-rich environment. Often environmental isolates of *Naegleria* are non-pathogenic (De Jonckheere 2002). Further culture-based analyses will be required to determine the characteristics of the amoeba in Nymph Creek.

PCR-based approaches to direct recovery and sequence analysis of rRNA genes and other taxonomically informative sequences from environmental samples provide a relatively rapid means of detection and enhance identification beyond that obtainable by cultivation and/or microscopy. Such advantages can facilitate monitoring of potential pathogens and enable more comprehensive analyses of amoebae in natural environments.

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