RESEARCH ARTICLE

Prevalence of Naegleria fowleri in Environmental Samples from Northern Part of India

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Abstract

Naegleria fowleri, the causative agent of Primary Amoebic Meningoencephalitis, is ubiquitously distributed worldwide in various warm aquatic environments and soil habitats. The present study reports on the presence of Naegleria spp. in various water bodies present in Rohtak and Jhajjar district, of state Haryana, India. A total of 107 water reservoirs were screened from summer till autumn (2012 and 2013). In order to isolate Naegleria spp. from the collected water samples, the water samples were filtered and the trapped debris after processing were transferred to non-nutrient agar plates already seeded with lawn culture of Escherichia coli. Out of total 107 water samples, 43 (40%) samples were positive by culture for free living amoeba after incubation for 14 days at 37°C. To identify the isolates, the ITS1, 5.8SrDNA and ITS2 regions were targeted for PCR assay. Out of total 43 positive samples, 37 isolates were positive for Naegleria spp. using genus specific primers and the most frequently isolated species was Naegleria australiensis. Out of 37 Naegleria spp. positive isolates, 1 isolate was positive for Naegleria fowleri. The sequence analysis revealed that the Naegleria fowleri strain belonged to Type 2.

Introduction

Free-living amoebae (FLA) such as Naegleria, Acanthamoeba, Vahlkampfia and Hartmannella are ubiquitously distributed worldwide in various aquatic and soil habitats. Many species of the genera Naegleria are known based on the analysis of their small subunit ribosomal deoxyribonucleic acid (SSU rDNA), large subunit ribosomal deoxyribonucleic acid (LSU rDNA) and the internal transcribed spacer (ITS) regions, including 5.8S rDNA [1]. Naegleria fowleri (N. fowleri) is the only species pathogenic to humans; it causes primary amoebic meningoencephalitis (PAM) [2–4]. PAM is rare but almost always fatal disease of the central nervous system (CNS) [4–7] often reported in healthy children and young adults after exposure to contaminated recreational, domestic or environmental water sources [4–6]. N. fowleri is thermophilic organism and can tolerate temperatures up to 45°C. Hence, these amoebae proliferate during warmer
months of the year when the ambient temperature is likely to be high. *N. fowleri* has been isolated from various aquatic habitats such as swimming pools, lakes, rivers, hot springs and tap water. Distribution of FLA including *N. fowleri* has been reported to range between 23% and 89% in some geographical locations [8–18]. Accidental exposure and infections occur primarily in children and young adults—as they are more active in aquatic activities (diving, jumping into water and underwater swimming) and are likely to come into direct contact with free living amoebae in contaminated water. The organism enters the nasal cavity and migrates through the olfactory neuro-epithelium to the central nervous system and causes a fatal infection that exhibits symptoms similar to acute bacterial meningitis.

There are at least 235 reported PAM cases worldwide [19], so the disease can be viewed extremely rare. But as it is almost always fatal, only about 5% of patients survive. Nearly two third cases of PAM have been reported from the United States of America (USA) alone and rest from the other parts of the world. So far, there are only 16 reported cases of PAM in India [20–35]. Out of 16 cases only 4 patients survived, of which 3 had no history of exposure to recreational water bodies, which makes it again suspicious whether these cases truly were *N. fowleri* infections. Of remaining 12 patients, 11 patients expired due to the disease, of which 4 were infants below 6 months of age. No conclusive information could be established for one patient as it was lost to follow up [31]. The presence of *N. fowleri* in swimming pools [36], pond water [37] and sewage canals [38] in India has been confirmed. It is believed that neurological infections by these free-living amoebas are misdiagnosed as well as under reported in India, probably due to inadequate information regarding their pathology or due to a very low autopsy rate.

It is also intriguing to note that above mentioned Indian reports are primarily case reports based on direct demonstration of *N. fowleri* in the CSF of the infected individual. However, no information was available on the source of infection as well as molecular detection of *Naegleria* species in environmental samples. In view of this the aim of the present study was to investigate the distribution of FLA with special reference to *N. fowleri* in selected water bodies in Rohtak and Jhajjar, of state Haryana, India. These two sites of the state were judiciously selected because of the fact that most of the clinical cases in North India were reported from these parts of the state.

**Material and Methods**

No ethical permission was required to carry out the study. The samples which were collected from the various water bodies didn't require any prior permission from civic agencies of the state. One of the co-author, Dr. Samander Kasuhik of Maharishi Dayanand University, Rohtak, Haryana had taken prior permission from the local leaders of the various villages for this study.

**Sampling sites and sample collection**

Collection of samples from selected water bodies in Rohtak and Jhajjar of state Harayana were done every 14 days from summer till autumn during the year 2012 and 2013. A total of 107 water reservoirs including ponds and lakes were screened. 5 samples from each of the 107 sites were collected by immersion of a 100 ml Schott Duran bottle into the upper 2 cm of the respective water body. Prior to sample collection, temperature and pH of the sampling sites were measured for each sample taken. The water samples were transported to the laboratory at ambient temperature to maintain the thermophilic amoeba in natural conditions and were processed within 2–4 hrs after sampling.

**Isolation and culturing of trophozoites**

In order to isolate free living amoeba *Naegleria* spp. from the collected water samples, further processing of the samples were done. The five, 100 ml volumes that were collected from
different parts of a single source was composited to make a 500 ml volume for the analysis. The samples were further filtered through a nitrocellulose membrane (pore size, 0.22 μm, Millipore, Fisher Scientific) [39]. Two to Eight nitrocellulose membranes were used to filter 500ml of water. The number of nitrocellulose membrane used for each sample varied on the basis of turbidity of the water sample. After filtration, the nitrocellulose membranes containing the trapped debris were flushed carefully in situ with 10 ml of sterile distilled water [40]. The water containing the debris after flushing was not possible to examine microscopically due to heavy content of the trapped soil and other impurities. However, it was further processed by centrifugation for 15mins at 1200 rpm. The pellet obtained was further re-suspended in 100 μl of supernatant.

Re-suspended pellets were gently pipetted onto a non-nutrient agar (NNA) plates prepared from PAGE amoeba saline overlaid with a lawn culture of Escherichia coli. The culture plates were sealed with parafilm and incubated at 37°C for up to 14 days [40, 41]. A temperature of 42–45°C is preferably used for the isolation of Naegleria species. However, to avoid the possible prohibitive effect of higher temperatures on the growth of certain non thermotolerant free-living amoebae a temperature of 37°C was selected in the present study [42].

Detection of Naegleria species

Daily examinations of all the culture plates were done up to 14 days using a light microscope (Olympus CX31). Cultures lacking morphological features of amoeba within 14 days were deemed negative and discarded. On initial observation it was found that the culture plates were colonized with mixed organisms and FLA like organisms. The culture plates which were suspected to be positive based on morphological characters were further subjected to clone establishment of the trophozoites by means of migration method [43]. Briefly, a block of agar containing small numbers of amoeba was transferred to a fresh NNA plate prepared from PAGE amoeba saline overlaid with a lawn culture of Escherichia coli. For documentation morphological characteristics were photographed.

PCR assays and DNA sequencing

The trophozoites were gently scraped from agar plates, re-suspended in PBS and subsequently pelleted by sedimentation at 1000g for 10 min. Total genomic DNA was extracted from amoeba trophozoites using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The positive control for the standardization of the PCR assay was isolated from N. fowleri culture obtained from American Type Culture Collection (ATCC no. 30894).

Fw1, (5’-GTGAAAACCTTTTTTCCATTTACA-3’) and RV1, (5’-AAATAAAAAGATTGCATTTGGAC CATTTGAAA-3’) were used as forward and reverse primers respectively for N. fowleri. Fw2, (5’-GAACCTGGCTAGGGATCATTT -3’) and RV2, (5’-TTTCTTTTCTCCCCCTTATTA -3’) were used as forward and reverse primers respectively for genus Naegleria. These primers are located respectively in the ITS1 and ITS2 regions [44]. Amplification was carried out with a PCR buffer containing 10 mMTrisHCl pH 9, 50 mM KCl, 1.5 mMmMgCl2, 0.1% Triton X 100 and 0.24 mM of each dNTPs, 0.4 μM of each primer, 1 unit of Taq DNA polymerase and 50–100 ng of genomic DNA in a final volume of 25μl. The PCR conditions comprised of initial denaturation for 3 min at 94°C, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s with a final elongation step at 72°C for 5 min. The expected PCR amplified product for Naegleria genus was 408–457 bp and for N. fowleri it was 310bp. The product was analysed by electrophoresis on 1.5% agarose stained with ethidium bromide and was visualized on a UV transilluminator. For the PCR assay described in this study a positive reaction was obtained with 9pg of purified DNA but not with the subsequent dilution tested of 0.9pg. The specificity
of the primers was checked with BLAST for other meningitis causing organisms like Cryptococcus neoformans, Haemophilus influenzae, Neisseria meningitidis, Staphylococcus aureus, Escherichia coli, Mycobacterium tuberculosis, Candida albicans and Acanthamoeba castellanii. No cross reactivity was observed with any of the above mentioned organisms.

Nucleotide Sequencing
The amplicons from 10 randomly selected isolates were gel purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). These were directly sequenced in both directions using respective forward and reverse primers using the BigDye Terminator sequencing v3.1 kit and an ABI 3130 XL genetic analyzer (Applied Biosystems, Foster City, CA, USA).

DNA chromatograms were examined using the BioEdit software version 7.1.3. The forward and reverse sequences were pair-wise aligned using ClustalW software and were manually refined to obtain a better consensus sequence. For nucleotide similarities a homology search was performed with BLAST against all eukaryotic nucleotide sequences archived in the GenBank database. Nucleotide residues corresponding to primer regions were excluded from the similarities search analyses.

Nucleotide Sequence Accession Number
The nucleotide sequences determined from this study were submitted to the Genbank and were assigned accession numbers KF700040, KF709528, KF709529, KF709530, KF709531, KF709532, KF709533, KF709534, KF709535 and KF709536.

Phylogenetic analysis
Phylogenetic analysis was carried out based on the internal transcribed spacers (ITS) and 5.8S sequences using the Molecular Evolutionary Genetics Analysis (MEGA) software. Phylogenetic trees were constructed using the neighbour joining with molecular distances estimated by the Kimura two-parameter model. The bootstrap procedure was then used to evaluate the robustness of each node.

Results
Microscopy and PCR assay result
A total of 107 water reservoirs including ponds and lakes were screened. The temperature, pH of these water reservoirs ranged between 24–31°C and 5.8–7.2 pH respectively (Table 1). Out of total 107 water samples tested, 43 (40%) samples were positive for free living amoeba by culture. Cultures were examined microscopically for the presence of morphological forms in all 43 isolates (Fig 1). These positive isolates were further subjected to PCR assay for detection of genus (Naegleria) as well as species (N. fowleri). Out of total 43 microscopically suspected positive samples, 37 (86%) isolates showed positive amplicons for genus Naegleria; where as 1 out of 37 (3%) amplicons was positive for N. fowleri (Fig 2).

Sequence analysis
The sequences of 10 randomly selected amplicons (ITS1-5.8S-ITS2) were analysed using ClustalW programme (Fig 3 and Table 2). Isolate no. 1 to 9 showed 99% homology with Naegleria australiensis (N. australiensis) except for isolate no. 3 and 9 which had shown 98% homology with N. australiensis. Isolate no. 10 had shown 99% homology with N. fowleri. The lengths (in bp) and differences in the ITS1, 5.8S, ITS2 and 28S rDNA sequences of representative isolates have been summarized in Table 3 and Fig 3.
Table 1. Overview of geographic sites sampled for *Naegleria* spp.

| Month | Sample ID | Geographical Coordinates of Sampling Sites | Temperature | pH  | PCR for *Naegleria* |
|-------|-----------|--------------------------------------------|-------------|-----|---------------------|
| March | R1CV      | 29°03'27"N 76°32'18"E                      | 29°C        | 7.6 | -                   |
|       | R2CV      | 29°03'43"N 76°31'46"E                      | 30°C        | 7.1 | +                   |
|       | R3LKM     | 29°02'19"N 76°28'34"E                      | 28°C        | 7.5 | -                   |
|       | R4LKM     | 29°02'38"N 76°28'26"E                      | 29°C        | 7.3 | -                   |
|       | R5LKM     | 29°02'43"N 76°28'40"E                      | 29°C        | 7.1 | -                   |
|       | R6CHV     | 29°01'37"N 76°30'01"E                      | 27°C        | 7.4 | -                   |
|       | R7CHV     | 29°01'53"N 76°30'17"E                      | 26°C        | 7.6 | -                   |
|       | R8CHV     | 29°01'59"N 76°30'09"E                      | 27°C        | 7.1 | -                   |
|       | R9BH     | 28°59'13"N 76°30'49"E                      | 29°C        | 5.8 | +                   |
|       | R10BH     | 28°59'10"N 76°31'05"E                      | 30°C        | 5.9 | +                   |
|       | R11BH     | 28°59'06"N 76°31'10"E                      | 29°C        | 5.8 | -                   |
|       | R12KIL    | 28°56'54"N 76°42'44"E                      | 30°C        | 6.2 | -                   |
|       | R13KIL    | 28°56'44"N 76°42'38"E                      | 29°C        | 6.5 | -                   |
|       | R14KIL    | 28°56'54"N 76°43'14"E                      | 29°C        | 6.3 | -                   |
|       | R15KIL    | 28°56'30"N 76°42'57"E                      | 29°C        | 6.7 | -                   |
| April | R16BHT    | 28°54'25"N 76°42'19"E                      | 30°C        | 5.9 | +                   |
|       | R17BHT    | 28°54'24"N 76°41'57"E                      | 29°C        | 6.0 | +                   |
|       | R18BHT    | 28°54'11"N 76°41'56"E                      | 29°C        | 5.8 | -                   |
|       | R19RHC    | 28°54'14"N 76°34'25"E                      | 28°C        | 7.1 | -                   |
|       | R20RHC    | 28°54'37"N 76°34'11"E                      | 29°C        | 7.2 | -                   |
|       | R21RHC    | 28°54'01"N 76°34'01"E                      | 28°C        | 6.8 | -                   |
|       | R22MOK    | 28°53'09"N 76°25'50"E                      | 28°C        | 6.4 | +                   |
|       | R23MOK    | 28°53'22"N 76°25'45"E                      | 29°C        | 6.7 | +                   |
|       | R24MOK    | 28°53'29"N 76°25'45"E                      | 29°C        | 5.9 | +                   |
|       | R25BOH    | 28°53'38"N 76°38'42"E                      | 28°C        | 6.2 | -                   |
|       | R26BOH    | 28°53'50"N 76°39'37"E                      | 29°C        | 6.4 | -                   |
|       | R27BAL    | 28°52'48"N 76°41'09"E                      | 29°C        | 6.7 | +                   |
|       | R28BAL    | 28°52'02"N 76°41'28"E                      | 30°C        | 6.9 | -                   |
|       | R29BAL    | 28°52'35"N 76°41'33"E                      | 29°C        | 6.7 | -                   |
|       | R30BAL    | 28°52'32"N 76°41'25"E                      | 29°C        | 6.3 | -                   |
|       | R31SKK    | 28°51'43"N 76°34'10"E                      | 28°C        | 6.6 | -                   |
|       | R32SKK    | 28°51'42"N 76°33'54"E                      | 30°C        | 6.8 | -                   |
|       | R33SKK    | 28°51'30"N 76°34'22"E                      | 29°C        | 5.9 | -                   |
| May   | R34KSV    | 28°51'30"N 76°40'43"E                      | 29°C        | 6.8 | +                   |
|       | R35KSV    | 28°51'42"N 76°40'16"E                      | 30°C        | 6.9 | +                   |
|       | R36KSV    | 28°51'18"N 76°39'58"E                      | 30°C        | 6.1 | +                   |
|       | R37MAV    | 28°51'01"N 76°36'17"E                      | 29°C        | 6.4 | -                   |
|       | R38MAV    | 28°50'38"N 76°36'13"E                      | 29°C        | 6.3 | -                   |
|       | R39GRW    | 28°49'12"N 76°33'46"E                      | 28°C        | 6.1 | +                   |
|       | R40KAL    | 28°49'40"N 76°23'22"E                      | 29°C        | 5.9 | -                   |
|       | R41KRT    | 28°48'11"N 76°37'23"E                      | 30°C        | 6.9 | +                   |
|       | R42KRT    | 28°48'11"N 76°36'53"E                      | 30°C        | 7.1 | +                   |
|       | R43KRT    | 28°47'48"N 76°37'01"E                      | 29°C        | 7.0 | +                   |
|       | R44KRT    | 28°47'50"N 76°37'25"E                      | 30°C        | 6.8 | -                   |
|       | R45SAP    | 28°46'28"N 76°46'31"E                      | 30°C        | 6.5 | -                   |
|       | R46SAP    | 28°46'47"N 76°46'33"E                      | 30°C        | 6.7 | +                   |
|       | R47SAP    | 28°46'40"N 76°45'41"E                      | 31°C        | 6.8 | -                   |

(Continued)
### Table 1. (Continued)

| Month | Sample ID | Geographical Coordinates of Sampling Sites | Temperature | pH | PCR for *Naegleria* |
|-------|-----------|-------------------------------------------|-------------|----|---------------------|
| June  | R48SAP    | 28°46'06"N 76°46'08"E                    | 30°C        | 6.5| +                   |
|       | R49SAP    | 28°46'09"N 76°46'25"E                    | 31°C        | 6.8| +                   |
|       | R50GRI    | 28°45'44"N 76°46'17"E                    | 30°C        | 6.3| -                   |
|       | R51GRI    | 28°45'30"N 76°46'20"E                    | 30°C        | 6.3| +                   |
|       | J52DIG    | 28°46'12"N 76°37'43"E                    | 29°C        | 5.9| -                   |
|       | J53DIG    | 28°46'17"N 76°38'03"E                    | 30°C        | 5.8| -                   |
|       | J54DIG    | 28°45'57"N 76°37'36"E                    | 29°C        | 6.0| +                   |
|       | J55DIG    | 28°45'50"N 76°38'10"E                    | 29°C        | 6.2| -                   |
|       | J56DIG    | 28°45'43"N 76°38'03"E                    | 30°C        | 5.9| +                   |
|       | J57DIG    | 28°45'33"N 76°37'42"E                    | 30°C        | 6.7| +                   |
|       | J58DHA    | 28°45'22"N 76°37'12"E                    | 30°C        | 6.1| -                   |
|       | J59DHA    | 28°45'14"N 76°37'20"E                    | 31°C        | 6.3| -                   |
|       | J60DHA    | 28°45'10"N 76°37'12"E                    | 30°C        | 6.6| -                   |
|       | J61GOC    | 28°44'02"N 76°35'41"E                    | 31°C        | 5.9| +                   |
|       | J62GOC    | 28°44'05"N 76°36'00"E                    | 30°C        | 5.8| +                   |
|       | J63SHE    | 28°43'17"N 76°36'34"E                    | 31°C        | 6.5| +                   |
|       | J64SHE    | 28°43'12"N 76°36'34"E                    | 29°C        | 6.0| -                   |
|       | J65SHE    | 28°43'05"N 76°36'30"E                    | 29°C        | 6.1| -                   |
|       | J66SHE    | 28°43'14"N 76°37'03"E                    | 30°C        | 6.3| +                   |
|       | J67SHE    | 28°42'47"N 76°36'37"E                    | 29°C        | 6.6| +                   |
|       | J68SHE    | 28°42'59"N 76°37'02"E                    | 31°C        | 6.4| +                   |
| July  | J69MAN    | 28°42'35"N 76°39'02"E                    | 29°C        | 5.9| -                   |
|       | J70MAN    | 28°42'46"N 76°38'34"E                    | 28°C        | 5.8| -                   |
|       | J71MAN    | 28°43'04"N 76°38'39"E                    | 29°C        | 6.0| -                   |
|       | J72MAN    | 28°43'03"N 76°39'06"E                    | 29°C        | 6.3| -                   |
|       | J73CHC    | 28°43'38"N 76°40'20"E                    | 30°C        | 6.1| -                   |
|       | J74CHC    | 28°43'42"N 76°40'30"E                    | 31°C        | 5.9| -                   |
|       | J75CHC    | 28°43'36"N 76°40'40"E                    | 30°C        | 6.3| -                   |
|       | J76CHC    | 28°43'34"N 76°40'46"E                    | 29°C        | 6.2| -                   |
|       | J77CHC    | 28°43'27"N 76°40'31"E                    | 30°C        | 6.7| -                   |
|       | J78CHC    | 28°41'01"N 76°36'56"E                    | 30°C        | 6.4| -                   |
|       | J79CHC    | 28°40'40"N 76°37'25"E                    | 29°C        | 5.9| -                   |
|       | J80BER    | 28°41'56"N 76°34'55"E                    | 30°C        | 6.0| +                   |
|       | J81BER    | 28°41'41"N 76°34'39"E                    | 31°C        | 5.8| +                   |
|       | J82BER    | 28°41'55"N 76°34'26"E                    | 31°C        | 6.3| +                   |
|       | J83BER    | 28°42'16"N 76°34'28"E                    | 30°C        | 6.7| -                   |
|       | J84BER    | 28°42'25"N 76°34'49"E                    | 31°C        | 6.2| +                   |
|       | J85BER    | 28°42'34"N 76°34'31"E                    | 29°C        | 6.4| +                   |
|       | J86BER    | 28°41'42"N 76°34'06"E                    | 30°C        | 6.8| -                   |
|       | J87BAG    | 28°41'30"N 76°31'51"E                    | 27°C        | 7.1| -                   |
|       | J88BAG    | 28°41'18"N 76°33'42"E                    | 27°C        | 7.0| -                   |
|       | J89WAZ    | 28°40'53"N 76°34'21"E                    | 28°C        | 7.2| -                   |
|       | J90WAZ    | 28°40'51"N 76°34'35"E                    | 27°C        | 6.9| +                   |
|       | J91WAZ    | 28°40'59"N 76°34'33"E                    | 27°C        | 7.0| +                   |
| August| J92MAJ    | 28°40'51"N 76°27'06"E                    | 24°C        | 7.2| -                   |
|       | J93MAJ    | 28°40'14"N 76°27'01"E                    | 25°C        | 7.0| +                   |
|       | J94MAJ    | 28°40'13"N 76°27'08"E                    | 24°C        | 7.1| -                   |
Phylogenetic analysis

The representative and reference isolates NGES 9, NGES 6, NGES 3 and *N. australiensis* GU597032 with similar sequences and length in the 5.8S (173 bp) were grouped together. NGES 4, NGES 5, NGES 7 and NGES 8 formed a clade to each other and showed similarity to the ITS1 (35bp). NGES 10 and *N. fowleri* (AY033619) formed a clade with each other showing similar sequences (Fig 4).

Table 1. (Continued)

| Month | Sample ID | Geographical Coordinates of Sampling Sites | Temperature | pH  | PCR for Naegleria |
|-------|-----------|--------------------------------------------|-------------|-----|------------------|
| J95MAJ| 28°40'18"N 76°27'08"E | 25°C | 7.1 | - |
| J96MAJ| 28°40'21"N 76°27'16"E | 24°C | 6.9 | - |
| J97MAJ| 28°40'15"N 76°27'32"E | 24°C | 6.9 | - |
| J98MAJ| 28°40'25"N 76°27'12"E | 25°C | 7.2 | - |
| J99MAJ| 28°40'28"N 76°27'59"E | 24°C | 7.0 | - |
| J100MAJ| 28°40'42"N 76°27'18"E | 26°C | 6.5 | - |
| J101MAJ| 28°39'49"N 76°26'54"E | 24°C | 6.6 | - |
| J102DUD| 28°41'08"N 76°29'25"E | 25°C | 7.0 | - |
| J103DUD| 28°40'55"N 76°29'04"E | 25°C | 7.2 | - |
| J104JHT| 28°35'52"N 76°39'07"E | 26°C | 6.9 | + |
| J105SIL| 28°32'52"N 76°41'07"E | 24°C | 7.1 | - |
| J106SIL| 28°32'40"N 76°40'52"E | 24°C | 6.8 | - |
| J107SIL| 28°32'53"N 76°40'45"E | 25°C | 7.2 | - |

* Sample was positive for *N. fowleri*

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**Fig 1.** Morphological observation of *Naegleria* -like cyst at 100X magnification. Zone of *Escherichia coli* clearing progressed and the number of cysts increased markedly.

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Discussion and Conclusions

Present study has primarily focussed to detect and identify the presence of pathogenic *N. fowleri* in the different water bodies in Rohtak and Jhajjar of state Harayana. Initially, identification of all the isolates of *Naegleria* -like species was based on the examination of morphological forms of the organism. The trophozoites of all the isolates showed erupted pseudopodia or lobopodia that actively moved in unidirectional manner while the cysts had uniform smooth thick double walls. All theses isolate exhibited flagella in distilled water except for four isolates. The detection of the flagellate stage was one of the important indicators for differentiation of *Naegleria* species from that of other FLA. All the 43 isolates showed luxuriant growth at 37°C.

In this study we used the PCR based detection in order to confirm the *Naegleria* species that were present in our environment. *N. fowleri* culture obtained from American Type Culture Collection (ATCC no. 30894) was used as the positive control for the standardization of the PCR assay. Two primer sets i.e. the *N. fowleri* specific and *Naegleria* genus specific were designed from the ITS1-ITS2 regions as already described by Pélandakis et al. [44]. The ribosomal ITS sequence has been reported to be a useful tool for detecting both inter- and intra-species differences of various microorganisms, such as *Cryptosporidium parvum* [45], *Naegleria* spp. [46] and *Vahlkampfia* spp. [47]. Within *Naegleria* spp. the differences between genus (inter-species) and species (intra-species of *N. fowleri*) are due to the sequence polymorphisms that occur at the ITS2 and ITS1 regions [40]. The ITS region has also been used for the identification of the new *Naegleria* isolates [11, 44].

In the current study *Naegleria* genus specific primer set successfully amplified the DNA template from 37 isolates, while the species specific primer set could amplify only one isolate of *N. fowleri* out of 37 isolates. The species specific primer set produced the amplicon consisting of 308bp, while the genus specific primer set produced amplicon consisting of 408–410 bp. Upon sequence analysis, *N. fowleri* (NGES10) identified in the study belonged to Type 2. Presence of the Pathogenic *N. fowleri* has been detected on all continents, except in Antarctica. So far, seven types have been reported in Europe, three in American and two in Asian continents [48]. Both type 3 and type 2 were common to Asian, American and European continents [19].
The other most frequently isolated species in this study was *N. australiensis*. It is pathogenic in mice but has not been isolated from human cases [49]. *N. australiensis* is also the closest
phylogenetic relative of *N. fowleri* (Fig 4) other than *N. lovaniensis* [1, 19]. It has been reported that *N. lovaniensis* is usually the most dominant species of *Naegleria* in warm water [50]. The data in the present study reveals that *N. australiensis* is the most frequently encountered species in the northern part of India especially Haryana. The reason for this feature observed warrants further investigation. *Naegleria* spp. were most frequently isolated during the summer season (April to July) (Fig 5), with the onset of monsoon the isolation rate reduced. The isolation frequency was high for water bodies with temperature ranging between 29–31°C (Table 1 and Fig 5). These were the noteworthy findings in the study.

In summary, our study is the first to use a PCR-based approach to screen and document the presence of *Naegleria* spp., in a variety of water bodies in the Northern state of India. Our results provide the evidence that *N. fowleri* is present in these natural water bodies. These FLA poses health risks to those people who use these aquatic systems for recreational activity and their day to day use. Considering the limited understanding of the ecology of *N. fowleri* in India, practical measures have to be taken for prevention and control of *Naegleria* infections. It includes better awareness of the disease within the medical community and educating general public with the help of civic authorities.

### Table 2. Result of DNA sequencing of the selected amplicons (NGES1-NGES10).

| Isolate | Accession No. | Closest phylogenetic species | Maximum identity | Reference Strain Accession No. |
|---------|---------------|------------------------------|------------------|-------------------------------|
| NGES 1  | KF700040      | *Naegleria australiensis*    | 99%              | GU597030                     |
| NGES 2  | KF709528      | *Naegleria australiensis*    | 99%              | GU597034                     |
| NGES 3  | KF709529      | *Naegleria australiensis*    | 98%              | GU597032                     |
| NGES 4  | KF709530      | *Naegleria australiensis*    | 99%              | GU597035                     |
| NGES 5  | KF709531      | *Naegleria australiensis*    | 99%              | GU597030                     |
| NGES 6  | KF709532      | *Naegleria australiensis*    | 99%              | GU597031                     |
| NGES 7  | KF709533      | *Naegleria australiensis*    | 99%              | GU597034                     |
| NGES 8  | KF709534      | *Naegleria australiensis*    | 98%              | GU597030                     |
| **NGES 10** | **KF709536** | **Naegleria fowleri**       | **99%**          | **AY033619**                 |

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### Table 3. Length and position of base in the ITS-5.8S-ITS2-28S sequence of the selected isolates (NGES1-NGES10).

| Isolate | Accession No. | Length and position of ITS1 sequence (in bp) | Length and position of 5.8S sequence (in bp) | Length and position of ITS2 sequence (in bp) | Length and position of 28S sequence (in bp) | Reference Strain Accession No. |
|---------|---------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|-------------------------------|
| NGES 1  | KF700040      | 34(1–34)                                    | 174(35–209)                                 | 99(201–309)                                 | 44(310–354)                                | GU597030                     |
| NGES 2  | KF709528      | 34(1–34)                                    | 174(35–209)                                 | 100(210–310)                                | 44(311–355)                                | GU597034                     |
| NGES 3  | KF709529      | 42(1–42)                                    | 173(43–216)                                 | 98(217–315)                                 | 44(316–360)                                | GU597032                     |
| NGES 4  | KF709530      | 35(1–35)                                    | 172(36–208)                                 | 100(209–309)                                | 44(310–354)                                | GU597035                     |
| NGES 5  | KF709531      | 35(1–35)                                    | 173(36–209)                                 | 100(210–310)                                | 44(311–355)                                | GU597030                     |
| NGES 6  | KF709532      | 32(1–32)                                    | 173(33–206)                                 | 99(207–306)                                 | 43(307–350)                                | GU597031                     |
| NGES 7  | KF709533      | 35(1–35)                                    | 173(36–209)                                 | 97(210–307)                                 | 47(308–355)                                | GU597034                     |
| NGES 8  | KF709534      | 35(1–35)                                    | 173(36–209)                                 | 98(210–308)                                 | 44(309–353)                                | GU597039                     |

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Fig 4. Neighbour-joining tree depicting the relationships between test isolates with amplicons (NGES1-NGES10) and reference strains of *Naegleria*. Numbers at the notes are percentage-bootstrapping values on 1000 replicates, and only values of > 50% are shown. GenBank accession numbers for reference sequences are indicated at the ends of the species designations.

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Author Contributions

Conceived and designed the experiments: BRM. Performed the experiments: AP SK YS. Analyzed the data: AP BRM. Contributed reagents/materials/analysis tools: BRM SK. Wrote the paper: AP SK BRM.

References

1. De Jonckheere JF. Molecular definition and the ubiquity of species in the genus Naegleria. Protist. 2004; 155(1):89–103. PMID: 15144061.
2. Schuster FL, Visvesvara GS. Free-living amoebae as opportunist and non-opportunistic pathogens of humans and animals. Int J Parasitol. 2004; 34(8):1001–27. PMID: 15313128.
3. Karanis P, Kourenti C, Smith H. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J Water Health. 2007; 5(1):1–38. PMID: 17402277.
4. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea. FEMS Immunol Med Microbiol. 2007; 50(1):1–26. PMID: 17428307.
5. Marciano-Cabral F. Biology of Naegleria spp. Microbiol Rev. 1988; 52(1):114–33. PMID: 3280964.
6. Marciano-Cabral F. Free-living amoebae as agents of human infection. J Infect Dis. 2009; 199(8):1104–6. PMID: 19302009. doi: 10.1086/597474.
7. Schild M, Gianinazzi C, Gottstein B, Muller N. PCR-based diagnosis of Naegleria sp. infection in formalin-fixed and paraffin-embedded brain sections. J Clin Microbiol. 2007; 45(2):564–7. PMID: 17121998.
8. Scaglia M, Strosselli M, Grazio VI, Gatti S, Bemuzzi AM, de Jonckheere JF. Isolation and identification of pathogenic Naegleria australiensis (Amoebida, Vahlkampfiidae) from a spa in northern Italy. Appl Environ Microbiol. 1983; 46(6):1282–5. PMID: 6660868.
9. John DT, Howard MJ. Seasonal distribution of pathogenic free-living amebae in Oklahoma waters. Parasitol Res. 1995; 81(3):193–201. PMID: 7770424.
10. Ettinger MR, Webb SR, Harris SA, McIninch SP, Garman CG, Brown BL. Distribution of free-living amoebae in James River, Virginia, USA. Parasitol Res. 2003; 89(1):6–15. PMID: 12474037.

Fig 5. Graph depicting the association between temperature of the sampling sites and isolation frequency of Naegleria spp. Y axis denotes the number of isolation and X axis denotes minimum and maximum temperature range of the sampling sites during the entire season (March to August).

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11. Sheehan KB, Fagg JA, Ferris MJ, Henson JM. PCR detection and analysis of the free-living amoeba *Naegleria* in hot springs in Yellowstone and Grand Teton National Parks. Appl Environ Microbiol. 2003; 69(10):5914–8. PMID:14532044.

12. Gornik K, Kuzna-Grygiel W. Presence of virulent strains of amphizoic amoebae in swimming pools of the city of Szczecin. Ann Agric Environ Med. 2004; 11(2):233–6. PMID:15627330.

13. Kilvington S, Gray T, Dart J, Morlet N, Beeching JR, Frazer DG, et al. Acanthamoeba keratitis: the role of domestic tap water contamination in the United Kingdom. Invest Ophthalmol Vis Sci. 2004; 45(1):165–9. PMID:14691169.

14. Tsvetkova N, Schild M, Panaiotov S, Kurdova-Mintcheva R, Gottstein B, Walochnik J, et al. The identification of free-living environmental isolates of amoebae from Bulgaria. Parasitol Res. 2004; 92(5):405–13. PMID:14760525.

15. Lorenzo-Morales J, Lindo JF, Martinez E, Calder D, Figueruelo E, Valladares B, et al. Acanthamoeba keratitis: the role of domestic tap water contamination in the United Kingdom. Invest Ophthalmol Vis Sci. 2004; 45(1):165–9. PMID:14691169.

16. De Jonckheere JF. Origin and evolution of the worldwide distributed pathogenic amoeboflagellate *Naegleria fowleri*. Infect Genet Evol. 2011; 11(7):1520–8. PMID:21843657. doi:10.1016/j.meegid.2011.07.023

17. Malhotra KK, Kakar PN, Pillay P, Pathak LR, Chuttani HK. Unusual presentation of primary amoebic meningoencephalitis—a serious diagnostic and therapeutic problem. J Trop Med Hyg. 1978; 81(6):113–5. PMID:660712.

18. Singh SN, Patwari AK, Dutta R, Taneja N, Anand VK. *Naegleria* meningitis. Indian Pediatr. 1998; 35(10):1012–5. PMID:10216726.

19. Jain R, Prabhakar S, Modi M, Bhatia R, Sehgal R. *Naegleria* meningitis: a rare survival. Neurol India. 2002; 50(4):470–2. PMID:12577098.

20. Shoff ME, Rogerson A, Kessler K, Schatz S, Seal DV. Prevalence of Acanthamoeba and other naked amoebae in South Florida domestic water. J Water Health. 2008; 6(1):99–104. PMID:17998610.

21. De Jonckheere JF. Origin and evolution of the worldwide distributed pathogenic amoeboflagellate *Naegleria fowleri*. Infect Genet Evol. 2011; 11(7):1520–8. PMID:21843657. doi:10.1016/j.meegid.2011.07.023

22. Kaushal V, Chhina DK, Ram S, Singh G, Kaushal RK, Kumar R. Primary amoebic meningoencephalitis due to *Naegleria fowleri*. J Assoc Physicians India. 2006; 54:327–9. PMID:16944619.

23. Singh P, Kochhar R, Vashishta RK, Khandelwal N, Prabhakar S, Mohindra S, et al. Amebic meningoencephalitis: spectrum of imaging findings. AJNR Am J Neuroradiol. 2006; 27(6):1217–21. PMID:16775267.

24. Rai R, Singh DK, Srivastava AK, Bhargava A. Primary amebic meningoencephalitis. Indian Pediatr. 2008; 45(12):1004–5. PMID:19129572.

25. Kaushal V, Chhina DK, Ram S, Singh G, Kaushal RK, Kumar R. Primary amoebic meningoencephalitis due to *Naegleria fowleri*. J Assoc Physicians India. 2008; 56:459–62. PMID:18822627.

26. Gupta N, Bhaskar H, Duggal S, Ghulaut PS, Kundra S, Arora DR. Primary amoebic meningoencephalitis: a case report. Indian J Pediatr. 2008; 75(2):121–2. PMID:18487831.

27. Angrup A, Chandey Y, Sood A, Thakur K, Jaryal SC. Primary amoebic meningoencephalitis due to *Naegleria fowleri*. J Inst Med. 2010; 32(2):56–59.

28. Khanna V, Khanna R, Hebbard S, Shashidhar V, Munckar S, Munim F, et al. Primary Ameobic Meningoencephalitis in an Infant due to *Naegleria fowleri*. Case Rep Neurol Med. 2011; 2011:782539. PMID:22937346. doi:10.1155/2011/782539
33. Gautam PL, Sharma S, Puri S, Kumar R, Midha V, Bansal R. A rare case of survival from primary amebic meningoencephalitis. Indian J Crit Care Med. 2012; 16(1):34–6. PMID: 22557831. doi:10.4103/0972-5299.94432

34. Yadav D, Aneja S, Dutta R, Maheshwari A, Seth A. Youngest survivor of Naegleria meningitis. Indian J Pediatr. 2013; 80(3):253–4. PMID: 22544673. doi:10.1007/s12098-012-0756-2

35. Sood A, Chauhan S, Chandel L, Jaryal SC. Prompt diagnosis and extraordinary survival from Naegleria fowleri meningitis: a rare case report. Indian J Med Microbiol. 2014; 32(2):193–6. PMID: 24713915. doi: 10.4103/0255-0857.129834

36. Gogate A, Deodhar L. Isolation and identification of pathogenic Naegleria fowleri (aerobia) from a swimming pool in Bombay. Trans R Soc Trop Med Hyg. 1985; 79(1):134. PMID: 3992632.

37. Gupta S. Isolation of Naegleria fowleri from pond water in West Bengal, India. Trans R Soc Trop Med Hyg. 1992; 86(1):46. PMID: 1566303.

38. Bose K, Ghosh DK, Ghosh KN, Bhattacharya A, Das SR. Characterization of potentially pathogenic free-living amoebae in sewage samples of Calcutta, India. Braz J Med Biol Res. 1990; 23(12):1271–8. PMID: 2136561.

39. Huang SW, Hsu BM. Survey of Naegleria from Taiwan recreational waters using culture enrichment combined with PCR. Acta Trop. 2011; 119(2–3):114–8. doi:10.1016/j.actatropica.2011.04.016 PMID: 21640066

40. Ithoi I, Ahmad AF, Nissapatorn V, Lau YL, Mahmud R, Mak JW. Detection of Naegleria species in environmental samples from Peninsular Malaysia. PLoS One. 2011; 6(9):e24327. PMID: 21915311. doi:10.1371/journal.pone.0024327

41. Barnett ND, Kaplan AM, Hopkin RJ, Saubolle MA, Rudinsky MF. Primary amebic meningoencephalitis with Naegleria fowleri: clinical review. Pediatr Neurol. 1996; 15(3):230–4. PMID: 8916161.

42. Gianinazzi C, Schild M, Zumkehr B, Wuthrich F, Nuesch I, Ryter R, et al. Screening of Swiss hot spring resorts for potentially pathogenic free-living amoebae. Exp Parasitol. 2010; 126(1):45–53. PMID: 20036656. doi:10.1016/j.exppara.2009.12.008

43. Gianinazzi C, Schild M, Wuthrich F, Ben Nouir N, Fuchslin HP, Schurch N, et al. Screening Swiss water bodies for potentially pathogenic free-living amoebae. Res Microbiol. 2009; 160(6):367–74. PMID: 19589386. doi:10.1016/j.resmic.2009.06.007

44. Pelandakis M, Serre S, Pernin P. Analysis of the 5.8S rRNA gene and the internal transcribed spacers in Naegleria spp. and in N. fowleri. J Eukaryot Microbiol. 2000; 47(2):116–21. PMID: 10750838.

45. Carraway M, Tzipori S, Widmer G. Identification of genetic heterogeneity in the Cryptosporidium parvum ribosomal repeat. Appl Environ Microbiol. 1996; 62(2):712–6. PMID: 8593074

46. De Jonckheere JF. Sequence Variation in the Ribosomal Internal Transcribed Spacers, Including the 5.8S rDNA, of Naegleria spp. Protist. 1998; 149(3):221–5. PMID: 23194635. doi:10.1016/S1434-4610(98)70030-6

47. Garstecki T, Brown S, De Jonckheere JF. Description of Vahlkampfia signyensis n. sp. (Heterolobosea), based on morphological, ultrastructural and molecular characteristics. Eur J Protistol. 2005; 41:119–127.

48. Moussa M, De Jonckheere JF, Guerlottte J, Richard V, Bastaraud A, Romana M, et al. Survey of Naegleria fowleri in geothermal recreational waters of Guadeloupe (French West Indies). PLoS One. 2013; 8(1):e55441. PMID: 23349880. doi:10.1371/journal.pone.0055441

49. Wang W, Wei F, Li J, Li N, Liu Q. Isolation and identification of Naegleria species from environmental water in changchun, northeastern china. Iran J Parasitol. 2014; 9(2):254–9. PubMed PMID: 25848933; PubMed Central PMCID: PMC4386047.

50. De Jonckheere JF. A century of research on the amoeboflagellate genus Naegleria. Acta Protozool. 2002; 41:309–342.