Naegleria fowleri from Pakistan Has Type-2 Genotype

Muhammad Aurongzeb 1,3, *Yasmeen Rashid 2, Syed Habib Ahmed Naqvi 3, Ambrina Khaaoon 4, Sadia Abdul Haq 4, Muhammad Kamran Azim 5, Imdad Kaleem 6, Shahid Bashir 7

1. Jamil-ur-Rahman Center for Genome Research, Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, 75270, Pakistan
2. Department of Biochemistry, University of Karachi, Karachi, 75270, Pakistan
3. Institute of Biotechnology & Genetic Engineering, University of Sindh, Jamshoro, Pakistan
4. Department of Research, Ziauddin University, 4/B, saharab-e-Ghalib, Block-6, Clifton, Karachi, 7500, Pakistan
5. Department of Biosciences, Mohammad Ali Jinnah University, Karachi, Pakistan
6. Department of Bioinformatics and Bioscience, COMSATS University (CUI), Islamabad, Pakistan
7. Neuroscience Center, King Fahad Specialist Hospital, Dammam, Saudi Arabia

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Abstract

Background: Primary amoebic meningoencephalitis (PAM) is an acute and fulminant CNS infection caused by Naegleria fowleri. Recreational activities and ritual ablation with contaminated warm fresh water are the main reason of PAM. Pakistan ranked the second most affected country, where most of the PAM incidences were reported from Karachi, Pakistan.

Methods: In May, 2019, a 28-yr-old suspected PAM patient came to the Imam Zain-ul-Abdin Hospital, Karachi. Biochemical and cytological investigations of patient’s CSF were carried out at Karachi Diagnostic Center and Molecular Biology Lab. Sequencing of Naegleria sp. specific (ITS) primer-based amplicons was performed from both patient’s CSF and water samples followed by multiple sequence alignment and phylogenetic studies.

Results: Biochemical and cytological investigations of patient’s CSF showed 5 mg/dl glucose, 240 mg/dl total protein and 2260/mm^3 TLC suggesting acute meningoecephalitis. PCR-based analyses of patient’s CSF and his residential tap water samples using Naegleria sp. specific (ITS) and N. fowleri specific primers revealed the presence of N. fowleri DNA. Nucleotide sequences of ITS primer-based amplicons from both patient’s CSF and water samples were submitted in GenBank under the accession numbers MT726981.1 and MT726226.1, respectively. According to phylogenetic analysis, N. fowleri isolate from Pakistan has shown the least node age of seven.

Conclusion: Here, for the very first time in Pakistan, N. fowleri genotype has been identified as type-2. Phylogenetic analysis showed that N. fowleri isolate from Pakistan is among the latest descendants, i.e., evolved later in life.

Keywords: Naegleria fowleri; Genotyping; Primary amoebic meningoencephalitis
Introduction

Naegleria fowleri is the causative agent of primary amoebic meningoencephalitis (PAM). It is a inhabitant of warm lakes, streams, spas, pools, domestic water reservoir and domestic water supplies (1-3). PAM cases are mostly observed in hot summers as N. fowleri proliferates rapidly at higher temperatures, i.e. 40-46 °C (4-6). Hundreds of PAM cases have been reported in last five decades; most probably due to global warming-related environmental changes (7). The WHO has also declared PAM as the second major cause of morbidity and mortality worldwide (8).

PAM incidences have been reported in many countries including America, Australia, Hong Kong, Thailand, Taiwan and China associated with a recent history of swimming in warm fresh water or direct exposure to contaminated tap water (1, 9-11). The clinical manifestations of PAM are quite similar to that of acute bacterial meningitis which makes PAM really hard to get differentiated from other bacterial meningitis. The resulting delayed diagnosis of PAM is one of main reasons of high mortality (1, 12-15). However, encephalopathic patients showing a triad of symptoms, i.e. fever, nausea and a low ESR should be urgently referred to lumbar puncture for confirmed diagnosis (16).

The first PAM case was reported in 2008 from Karachi, Pakistan (1). An obvious increase in PAM incidences has been reported during the last few years. In USA, N. fowleri infection has been identified generally in children of less than 14 years of age. In contrast, most of the PAM patients in Pakistan were adults having 26–45 years of age. This prominent difference indicates somewhat unique N. fowleri strain in Pakistan which needs to be characterized in detail (17-19).

Despite of morphological similarities among N. fowleri isolates, eight distinct N. fowleri genotypes have been characterized on the basis of differences in ribosomal internal transcribed spacers, including 5.8S rDNA. The ITS and 5.8S rDNA sequences will be of additional help in describing new Naegleria spp. in future (20). According to the previous studies, N. fowleri genotypes are unequally dispersed in different continents where genotypes 1, 2 and 3 are found in America, genotypes 2, 3, 4, 5, 6, 7 and 8 in Europe and genotypes 2 and 3 occur in Asia. Out of these eight genotypes, only types 1, 2, 3 and 5 are clinically significant (1, 21). Annotation of particular pathogenic genotype is likely to assist the development of a potential genotype-specific vaccine or drug against it. Additionally, different geographical distribution of diverse genotypes made them important epidemiologic marker that can trace the source of infection in a particular population (22, 23). N. fowleri type-2 genotype has been reported from many Asian countries but has not been testified yet in Pakistan (15, 24, 25).

The main aim of the present study was to perform genotyping of Pakistani N. fowleri isolate and reveal its phylogenetic relationship with other N. fowleri isolates.

Materials and Methods

A 28 yr-old suspected PAM patient came to the Imam Zain-ul-Abdin Hospital, Karachi in 2019. Biochemical and cytological investigations of patient’s CSF were carried out at Karachi Diagnostic Center and Molecular Biology Lab.

Ethical approval and consent to participate

This study was approved by the Ethics Committee of Karachi Diagnostic and Molecular Biology Lab (EC Ref No: RECNF02). The patient was unconscious that is why the...
informed consent was acquired in written form from his elder brother.

**Biochemical, cytological and microscopic analysis of patient’s CSF sample**

Cytological analysis of fresh CSF sample was carried out using Sysmix KX 21N Hematology analyzer. For further analysis, the CSF sample was centrifuged at 2000×g for 15 min at room temperature (26, 27). The supernatant was analyzed for the estimation of Glucose and overall protein content. The pellet was resuspended in 200 µl of supernatant followed by 30 min incubation at 37 °C. Resuspension was used both for the *N. fowleri* detection by direct microscopy and *N. fowleri* cultivation on attenuated *E. coli* (ATCC number 25922) containing non-nutrient agar plate (28-30).

**Detection of *N. fowleri* DNA in patient’s CSF and residential tap water**

The CSF sample was centrifuged at 10,000×g for 10 min. The sediment was subjected to DNA extraction using the QIAamp DNA Mini Kit (Qiagen Inc., USA). Tap water sample (1000 ml) was collected in a sterile bottle from patient’s residency and processed within 12 hours. The water sample was filtered through nitrocellulose membrane (MS®MCE Gridded Membrane Filter) having 0.45 µm pore-size to trap *N. fowleri*. The nitrocellulose membrane was aseptically removed from filtration apparatus, cut into small pieces and placed in 50-ml sterile falcon tube. Wash buffer was prepared by adding 10 µl Tween 20 to 5 ml of AE buffer (QIAamp DNA Mini Kit) and immediately transferred in membrane containing falcon tube followed by vortex for 3 minutes. Rest of the protocol was same as recommended in QIAamp DNA Mini Kit (Qiagen Inc., USA). Genomic DNA was stored at -20 °C.

*N. fowleri* PCR-based detection

Identification of *N. fowleri* among other *Naegleria* spp. based on cellular morphology is difficult so PCR-based detection was performed. As reported previously (31-34), amplification assays were performed in a total volume of 25 µl, containing 9.5 µl of ddH2O, 0.5µl of each primer (10µM), 10 µl Green Master Mix (Promega, USA), and 5.0µl of template genomic DNA extracted from the CSF samples. For detection of *Naegleria* spp. *NfITS1*-F and *NfITS1*-R primer set was used. Whereas, for specific detection of pathogenic *N. fowleri* in the CSF samples, *NaeglF1925* and *NaeglR344* primer set was used.

\[
\begin{align*}
Nf\text{-}ITS1\text{-}F & \quad 5`\text{-GAACCTCGG\,-} \\
Nf\text{-}ITS1\text{-}R & \quad 3` \\
Nf\text{-}ITS2\text{-}R & \quad 5`\text{-TTTCTTTTCTCCCCTTATTA}\, \text{3` (35)} \\
NaeglF1925 & \quad 5`\text{-GTGCTGAAACCTAGCTATTGTAACTCAGT}\, \text{3`} \\
NaeglR344 & \quad 5`\text{-CACTAGAAAAAGCAAACCTGAAAGG}\, \text{3` (36)} \\
\end{align*}
\]

Followed by a prolonged denaturation i.e. 95 °C for 5 min, amplification reactions with primer pairs were performed in 40 cycles of denaturation at 95 °C for 3 seconds, annealing at 53 °C for 30 seconds and extension at 72 °C for 30 seconds. A final extension at 72 °C for 5 min was included. Amplified product was visualized in a 2 % agarose gel (31).

**Sequence Analysis and Genotyping**

The purified PCR product of internal transcribed spacer-1 (ITS1), 5.8S rRNA gene, internal transcribed spacer-2 (ITS2) region was sequenced by both ITS-1 and ITS-2 primers using ABI PRISM 3730 Genetic Analyzer (Applied Biosystems Inc., USA). The reaction mixture was prepared by mixing 7 µl of purified PCR product, 0.5 µl primer (10 µM), 1.25 µl buffer and 1.5 µl Big Dye Terminator (Applied Biosystem Inc., USA). The sequencing reaction was subjected to 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes in thermal cycler. Ethanol precipitation was carried out to re-

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move the left over fluorescent dye followed by washing of pellet with 70% ethanol. Each pellet was resuspended in 10 μL of HiDi Formamide (Applied Biosystem Inc., USA), heat denatured for 5 min at 95 °C and quickly chilled on ice for 3 minutes followed by DNA sequencing. The nucleotide sequences of Nf-ITS regions from patient’s CSF and tap-water acquired in this study has been submitted to GenBank with the accession numbers MT726981.1 and MT726226.1, respectively.

Genotyping was performed using previously reported method (10). Briefly, reference sequences of eight existing genotypes consisting of ITS-1, 5.8S rDNA and ITS-2 regions of Naegleria spp. including AY376149, X96564, X96562, AJ132030, AJ132028, FR875287, X96563, and FR875288 were retrieved from GenBank and aligned with MT726981.1 and MT726226.1 sequences using MEGA-X program (37). Furthermore, phylogenetic analysis was performed to delineate the evolutionary relationship of N. fowleri isolate from Pakistan with other N. fowleri strains isolated so-far using Maximum Likelihood Tree construction method on Mega-X program (37-39).

Results

Biochemical and cytological investigations of PAM patient’s CSF

The patient was the resident of Liaquat-abad town and had no previous history of any recreational activity. He might have used contaminated water during ritual ablution or bath. His CSF sample was received in Karachi Diagnostic Center and Molecular Biology Lab for the detection of PAM. CSF analysis showed a white blood cell (WBC) count of 2260/mm³ including 75% neutrophils and 25% lymphocytes. Analysis of the fresh CSF indicated glucose at a concentration of less than 5 mg/dl and proteins at a concentration of more than 240 mg/dl.

Direct microscopy of patients' fresh CSF sample

Direct microscopy of fresh CSF smear found alive motile amoebic cells with pseudopodia in the CSF sample (Fig. 1). The continuous change in cell morphology and formation of pseudopods suggested the trophozoite state of amoeba. The trophozoites were approximately 12-15 μm in size. The crawling amoeba was observed to move rapidly with ~1 μm/s speed using eruptive pseudopods.

Fig. 1: Direct microscopy of patients fresh CSF sample showing the presence of motile amoeba (depicted by arrows) using 40X
**PCR-based detection of N. fowleri**

PCR using Nf-ITS1-F_Nf-ITS2-R primer pair amplified a 410bp fragment of *Naegleria* specie ITS (including ITS1, 5.8S rDNA and ITS2) region which confirmed the presence of *Naegleria* species in general (Figure 2A). However, the presence of pathogenic *N. fowleri* in the CSF was confirmed using NaeglF1925_NaeglR344 set of primers; which showed a 153bp amplicon consisting of a region of 18S rDNA in pathogenic *N. fowleri* (Fig. 2B).

![PCR amplicons](image)

**Fig. 2**: PCR amplicons obtained using *N. fowleri* specific (NaeglF1925: NaeglR344) (A) and *Naegleria* spp. specific (Nf-ITS1-F: Nf-ITS1-R) primer pairs (B). In part A, lane 1-3: Marker, CSF, Tap water. In part B, lane 1-4: Tap water, CSF, blank, Marker

**Isolation of N. fowleri on non-nutrient agar (NNA)**

Alternate day examinations of the NNA culture plate was done up to 10 days using a light microscope with 10X magnification (OPTIKA, B-382 PLi, Italy). The trophozoite stage was observed after third day of culture in the CSF sample.

**Sequence Analysis and Genotyping**

The genotyping of the isolated *N. fowleri* was elucidated using ITS-1 and 5.8S rRNA gene region. The ITS-1 region is comprised of 85bp which is divided into R1 (16bp), R2 (28bp), M1 (15bp) and M2 (27bp) regions. Sequence analysis showed that repeats R1 and R2 were absent in ITS-1 region of both patient’s CSF and water samples, i.e. MT726981.1 and MT726226.1, respectively. However, presence of two main regions i.e. M1 and M2 having 42bp length and C>T transition at position 31 in 5.8S rRNA gene sequence was observed (Fig. 3).
Fig. 3: Multiple sequence alignment of the nucleotide sequences of ITS and 5.8S rDNA from eight existing \textit{N. fowleri} genotypes including same region from Pakistani \textit{N. fowleri}. One copy of each M1 and M2 region of ITS-1 is apparent while C>T transition at location 31 of 5.8S rDNA is also shown (yellow box).

**Evolutionary relationships with other \textit{N. Fowleri} genes**

Phylogenetic analysis of MT726981.1 and MT726226.1 sequences was performed by aligning them with other 36 available \textit{N. Fowleri} ITS-1, 5.8S, ITS-2 region-based sequences. Pakistani \textit{N. fowleri} isolate showed closed homology with eleven other \textit{N. Fowleri} isolates including nine from Asia and three from USA (Fig. 4).
Discussion

In Karachi, Pakistan, PAM remains an overwhelming CNS infection connected with warm freshwater exposure since last decade. Having a population size of ~27 million, Karachi is the largest city, industrial hub, financial capital and commercial center of Pakistan. The geographical changes especially recurrent heat shocks seem to be the cause of N. fowleri outbreaks in this city. Considering the rapid onset and high mortality rate of this deadly disease, potential vaccine, early diagnosis and effective treatment is very crucial. PAM can be confirmedly diagnosed by direct microscopy, amoebic cultivation on NNA plated with E. coli and PCR. Selective isolation of N. fowleri requires culture temperature between 42-45 °C at which the growth of other amoebae is strictly suppressed. Although N. lovaniensis is exceptional Naegleria species that can grow at 42-45 °C but being non-pathogenic it cannot be present in CSF. Generally, confirmed diagnosis of PAM is carried out from patients’ CSF using Naeg and ITS primer-based PCR assays (2, 21, 40-42).

The present study was focused on detection and isolation of Pakistani N. fowleri isolate from patient’s CSF and his residential tap water in order to reveal its yet unknown genotype as this could help in designing genotype-specific vaccine and drugs in future. The biochemical and cytological analyses showed an overall low concentration of glucose, high concentration of total protein and a high value of total leucocyte count with increased per-

Fig. 4: Phylogenetic analysis of 38 N. Fowleri ITS-1, 5.8S, ITS-2 region-based sequences including those from PAM patient’s CSF and water samples using Maximum Likelihood Tree construction method (39)
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percentage of neutrophils. Similar findings have also been reported in other types of meningitis suggesting inadequacy of these analyses in discriminating PAM from other meningitis. However, direct microscopy of the CSF sample detected motile amoeba. Additionally, PCR analysis of both samples using ITS- and Naeegl-primers also showed the presence of N. fowleri DNA.

Our primary finding was that the genotype of Pakistani N. fowleri isolate is type-2; the predominant type in Asia. Additionally, Pakistani N. fowleri isolate is among those which originated later in life. According to the calculated node ages, N. fowleri isolate from Philippine, USA is thought to be the ancestral isolate. Whereas, N. fowleri isolate from Pakistan is among the latest descendants showing a node age of seven.

Conclusion

Molecular genotyping studies revealed that Pakistani N. fowleri isolate belongs to type-2. The identification of N. fowleri in patient’s residential tap water confirmed the source of infection. Parallel tap water sample analysis showed the presence of coliform along with low chlorine level (data not shown). The presence of coliform strongly indicates sewage contamination in tap water. The Karachi citizens are at high risk as they use the same tap water in raw form for routine ritual ablation. Preventive measures including proper chlorination and water/sewerage pipeline fixture should be taken by Karachi Water and Sewerage Board (KWSB) on urgent basis.

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Conflict of interest

There is no conflict of interest.

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