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Genotyping of *Acanthamoeba* spp. isolated from patients with granulomatous amoebic encephalitis

Sir,

Free-living amoebae of genera *Acanthamoeba, Balamuthia* and *Naegleria* are the most prevalent protozoa found in ecological environments and have been implicated in causing human infections. *Acanthamoeba* is implicated in causation of granulomatous amoebic encephalitis (GAE), a life-threatening disease primarily occurring in immunocompromised individuals, *Acanthamoeba* keratitis (AK), a painful sight-threatening infection of the cornea mainly in immunocompetent individuals and rarely cutaneous disease[1]. *Acanthamoeba* enters the central nervous system either by inhalation of airborne cysts or by haematogenous route through a skin lesion. GAE is usually associated with immunocompromised states, including human immunodeficiency virus and organ transplant[2], but has also been reported from immunocompetent individuals[3]. There are around 500 cases of GAE worldwide with <10 per cent survival rate. GAE begins as subclinical or non-specific entity, but unless diagnosed at an early stage, it usually culminates in the death of the patient[4]. The majority of GAE infections are diagnosed post-mortem. The ante-mortem diagnosis of GAE can be made initially by radiological examination including computed tomography (CT) and magnetic resonance imaging (MRI). Cerebrospinal fluid (CSF) and brain abscess may be examined for the presence of *Acanthamoeba* by microscopy, culture and molecular methods[5]. A number of genotypes (T1-T20) of *Acanthamoeba* have been identified, of which human infections are associated with a few only[6]. Thus, it is important to study the molecular epidemiology of *Acanthamoeba* infections in different geographical areas to know the prevalent genotypes. The most recently proposed method for molecular identification of *Acanthamoeba* is *18S rDNA* gene sequence analysis[7,8]. *Acanthamoeba* genotypes are differentiated by five per cent or greater sequence dissimilarity between isolates[7,9]. Genotypes T3, T4, T5, T6, T11 and T15 are confirmed to be causative agents of AK whereas genotypes T1, T3, T4, T10 and T12 are implicated in GAE[7,10,11]. T4 is the most predominant genotype found in both clinical and environmental isolates[12].

To cut short the time and cost in sequencing the entire gene, *Acanthamoeba* can also be genotyped by targeting one of the highly variable region, designated diagnostic fragment 3 (DF3) and located within genus-specific 18S rDNA[8,13]. This segment includes portions of *18S rDNA* conserving stem 29 and all of 29-1[14]. The present study was undertaken to genotype *Acanthamoeba* spp. isolated from patients diagnosed with GAE in a tertiary care hospital in north India. This retrospective study was conducted in the department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. Briefly, five isolates of *Acanthamoeba* obtained from patients with GAE collected over a period of the past 10 years from July 2007 to July 2017 and maintained on Non-Nutrient agar (NNA) medium overlaid with *Escherichia coli* were used in the study. These were further axenized in the peptone yeast dextrose (PYD) medium for genetic characterization. DNA was extracted from axenically cultured *Acanthamoeba* isolates by phenol-chloroform-isoamyl alcohol method using UNSET lysis buffer[15]. Extracted DNA was subjected to PCR amplification of DF3 of *18S rDNA* using genus-specific primer JDP1 (5'-GGCCCAGATCGTTTACCGTGAA-3') and JDP2 (5'-TCTCACAAGCTGCTAGGGAGTCA-3')[8]. PCR cycles included initial denaturation at 95°C for seven min followed by 39 cycles of denaturation at 95°C for one min, annealing at 60°C for one min, extension at 72°C for two min and final extension at 72°C for 10 min.

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Table. Genotypic identification of *Acanthamoeba* spp. obtained from granulomatous amoebic encephalitis patients and their clinical characteristics

ID	Source of origin	Genotype	Underlying immune state	Age (yr/sex)	Time of diagnosis	Final outcome	GenBank accession number
AC 8	CSF	T4	Immunocompetent	19/female	Post-mortem	Died	KX675338
AC 14	CSF	T4	Acute on chronic malnutrition	3/male	Ante-mortem	Survived	KX675339
AC 2	CSF	T11	Immunocompetent	10/male	Ante-mortem	Survived but CT after 2 months still showed persistence of mass-like lesion. Final outcome not known	KX675340
AC 24	CSF	T11	Immunocompetent	22/male	Post-mortem	Died	KX675341
AC 27	Brain abscess	T10	Acute myeloid leukaemia on treatment	19/male	Ante-mortem	Died after 6 months	KX675337

CT, computed tomography; CSF, cerebrospinal fluid

Amplified DNA products were separated by 1.5 per cent agarose gel electrophoresis stained with ethidium bromide and visualized under ultraviolet light using an image analyzer. Direct sequencing of the PCR product was done with an ABI 310 automated fluorescent sequencer (Applied Biosystems, USA) with the primer 892C (5'-GTCAGAGGTGAAATTCTTGG-3') to determine the DNA sequence of DF3 of *18S rDNA*. The nucleotide similarity search was performed by BLAST search of sequenced amplicons in GenBank database (*http//www.ncbi.nlm.nih.gov/blast*). CLUSTAL X was used to determine multiple sequence alignments. Neighbour-joining distance trees were prepared using MEGA6 software (*https://www.megasoftware.net/*). Bootstrap values were based on 1000 replicas. Sequences obtained from this study were submitted to GenBank with accession numbers (*http//www.ncbi.nlm.nih.gov/genbank*) shown in Table.

All five isolates shared >96 per cent identity with *Acanthamoeba* spp. However, none of our isolates had 100 per cent sequence identity to any of the available strains in GenBank, suggesting that polymorphism exists in these isolates (Table). Two of the five isolates from patients with GAE were determined to be T4 genotype (*A. castellanii*), two were T11 genotype (*A. hatchetti*) and one was T10 genotype (*A. culbertsoni*). The phylogenetic tree reconstructed using neighbour-joining method with the partial sequence of DF3 region of *18S rRNA* gene is shown in Fig. 1.

All the patients were young adults or children with the age range of 3-22 yr. All except one were male. Three patients with GAE were immunocompetent while one had underlying acute myeloid leukaemia and one child had acute on chronic malnutrition. Diagnosis

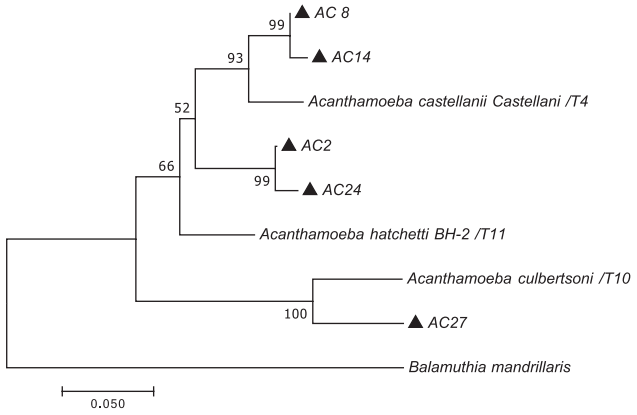

Fig. 1. Phylogenetic tree constructed with the neighbour-joining method using nucleotide sequences of diagnostic fragment 3 region of *18S rDNA* gene of *Acanthamoeba* granulomatous amoebic encephalitis isolates. Isolates from this study are shown in bold face and triangle shape. AC, *Acanthamoeba* isolates.

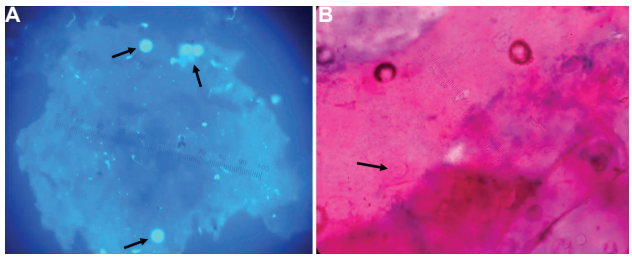

Fig. 2. *Acanthamoeba* cysts in brain abscess stained with (**A**) calcofluor-white stain, and (**B**) haematoxylin and eosin stain visualized by microscopy (×1000).

of GAE was made post-mortem in two patients while three patients were diagnosed ante-mortem of whom only one patient survived. The haematoxylin and eosin and calcofluor while stained slides of brain abscess of a patient with *Acanthamoeba* cysts are shown in Fig. 2. This patient was infected with T4 genotype and

had underlying acute on chronic malnutrition. Final outcome of one patient infected with T11 genotype could not be known, but CT scan done on this patient two months after improvement and discharge still showed a mass-like lesion. The only patient infected with T10 genotype died after six months (Table).

Though 20 genotypes of *Acanthamoeba* have been identified, but only a few genotypes are associated with clinical disease. Moreover, clinical course and outcome of disease may be influenced by the infecting genotype[16]. In our study, genotypes T4 and T11 were present in two patients each while T10 was identified in one. A study on GAE patients from India reported only T4 genotype[17]. Studies from other parts of the world have also reported genotype T4 to be the predominant genotype while less frequent genotypes were T1, T2, T5, T10 and T18[18-20]. Genotype T11 reported in our study has not been previously reported from GAE patients though it has been isolated from keratitis patients[21]. We have previously reported T3 and T4 genotypes from water sources[22].

In this study, the number of isolates was too less to draw some definite conclusion about the association of disease outcome with specific genotype. However, the information generated on these five cases can add to the existing literature of disease association with genotypes. More isolates need to be studied for understanding the association of various genotypes with disease spectrum.

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