18S rDNA sequencing aided diagnosis of *Acanthamoeba jacobsi* keratitis - A case report

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Identification of Acanthamoeba cysts and trophozoites in cases of keratitis is traditionally done with microbiological techniques such as smear examination with 10% potassium hydroxide (KOH) and culture. Double walled cyst with hexagonal inner wall is characteristic of Acanthamoeba. We report a unique case of a 9 year old boy who presented with dense anterior corneal stromal infltration, which on smear examination showed atypical double walled spherical cysts, leading to a diagnostic dilemma. An 18S rRNA gene-based PCR done on the growth on culture, subsequently identifed a rarely reported species of Acanthamoeba. The patient was advised combination therapy with polyhexamethylene biguanide (PHMB 0.02%) and chlorhexidine (0.02%) eye drops. Three weeks post treatment, the keratitis resolved with scarring and vascularisation and visual acuity improved to 20/60. At 8 weeks follow up Best corrected visual acuity further improved to 20/30 with contact lens.

Key words: Acanthamoeba, gene sequencing, keratitis

Acanthamoeba are parasites commonly present in the natural environment and are a frequent cause of keratitis.[1] The first case of *Acanthamoeba* keratitis was reported in a contact lens wearer in 1974.[2] The prevalence of *Acanthamoeba* keratitis (AK) ranges from 1 per 10,000 to 1,000,000 as per various studies.[3] The clinical presentation of *Acanthamoeba* keratitis is varied and microbiological analysis is a useful tool in definite identification of the organism. Traditional microbiological techniques to identify *Acanthamoeba* species in corneal scrapings comprise of 10% potassium hydroxide (KOH) mount which characteristically shows double walled cysts of *Acanthamoeba* with hexagonal inner wall, Gram's stain and culture on non-nutrient agar (NNA) with *E. coli* overlay. However, an 18S rDNA gene-based PCR test, highly specific for the genus *Acanthamoeba*, is a useful tool for molecular confirmation of *Acanthamoeba* keratitis as described by Schroeder *et al*.[4] We report a case of *Acanthamoeba* keratitis that showed atypical morphology of cysts in direct microscopy of the corneal scrapings and application of molecular test subsequently helped identify a rarely reported species of *Acanthamoeba.*

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Case Report

A 9-year-old male, resident of North India presented with complaints of diminution of vision, pain, photophobia, redness, and watering in the right eye since 3 months. There was no history of trauma or use of contact lenses. His Best Corrected Visual Acuity (BCVA) in the right eye was ability to perceive Hand Movements Close to Face (HMCF) and in the left eye was 20/20. On anterior segment examination, the upper palpebral conjunctiva in both eyes showed papillae with congestion. The cornea had dense anterior stromal infiltration with an epithelial defect (7 mm × 5 mm), surrounding stromal oedema and vascularization. [Fig. 1a] Anterior chamber, lens, and fundus details were indiscernible in the right eye. B scan ultrasonography of the right eye revealed normal lens and posterior segment status. The left eye examination was essentially within normal limits.

The patient was diagnosed as a case of microbial keratitis elsewhere and was on 4th generation fluoroquinolone and antifungal eyedrops on presentation to us since the past 8 weeks. Corneal scraping was done at our hospital which did not reveal any organism on microsopic smear examination with 10% potassium hydroxide (KOH), Gram, and Giemsa stains. The patient was then given a drug holiday where in only tear supplements, cycloplegic and bacteriostatic chloramphenicol (0.5%) eye drops four times a day were prescribed. Forty-eight hours post drug holiday, rescraping was done. Smear examination showed double-walled spherical cysts in KOH preparation and Gram's stain [Fig. 2]. Although the cysts did not represent the typical hexagonal inner walled cysts of *Acanthamoeba*, a presumptive diagnosis of *Acanthamoeba* keratitiswas made based on clinical presentation. The patient was advised monotherapy with 0.02% polyhexamethylene biguanide (PHMB) eye drops hourly, 1% atropine eye drops 3 times a day and tear supplements 3 times a day. One week post treatment with PHMB (0.02%) eye drops, the patient did not show clinical improvement [Fig. 1b]. Eyedrop chlorhexidine (0.02%) 1 hourly was then added to the treatment. Rescraping was done at this stage and smear examination again showed double-walled spherical cysts.

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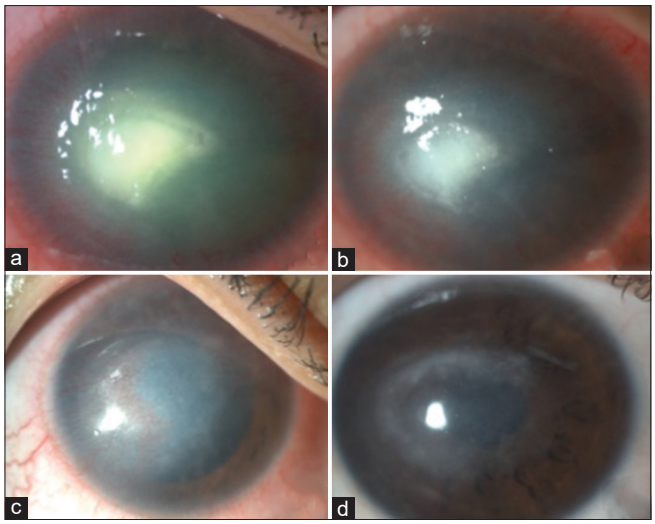

Figure 1: Clinical picture (a) Day of presentation; dense anterior stromal infiltration with an epithelial defect and surrounding stromal oedema and vascularisation (b) status quo post 1 week of treatment with PHMB (0.02%) eyedrops (c) commencement of peripheral healing at 3 weeks post addition of chlorhexidine (0.02%) eyedrops (d) complete healing and vascularization of ulcer at 6 weeks post treatment with PHMB (0.02%) and chlorhexidine (0.02%) eyedrops

As the morphology of the cysts resembled cysts of *Dictyostelium polycephalum* reported in earlier case reports, additional corneal scrapings of the patient were sent to L. V. Prasad Eye Institute (LVPEI) for a PCR test.[5] Corneal scrapings inoculated on non-nutrient agar plate with *E. coli* overlay were also sent. DNA was extracted from the corneal scraping (DNeasy® plant mini kit, Qiagen, Cat No./ID: 69104, Germany) and was subjected to 18S rDNA based PCR using earlier described JDP 1 and 2 primers specific to the genus *Acanthamoeba*.[4] *Acanthamoeba* DNA was detected. After 48 hours of incubation, the non-nutrient agar plate with *E. coli* overlay showed growth of trophozoites and cysts resembling *Acanthamoeba.* The isolate was made axenic and in order to determine species, previously described primers for free-living amoeba were used for 18S rDNA based sequencing.[6] Chromatogram analysis, as well as the sequences obtained using forward and reverse primers targeting 18S rDNA were assembled using DNA star laser gene SeqManpro 7.1 software. BLAST algorithm with default parameters were used to identify closest neighbors in NCBI nucleotide sequence database which confirmed the organism to be *Acanthamoeba jacobsi* (99% homology) with the query coverage of assembled sequence being 100 and the "e – value" as 0.0.

Clustal W multiple alignment was performed through BioEdit software to align the sequences of the clinical isolate and the reference strains of closely related species. The phylogenetic relationship of the aligned sequences was determined using neighbor joining algorithm of MEGA software version 6.06 with 1000 boot strap replications [Fig. 3]. The sequence was deposited in the NCBI data base under accession number MK673930.

At 3 weeks, the patient showed clinical improvement with decrease in photophobia and peripheral scarring and vascularization of the ulcer [Fig. 1c]. The frequency of eye drops chlorhexidine (0.02%) was reduced to 4 times a day and eye drops PHMB (0.02%) was prescribed 8 times a day. By 8 weeks,

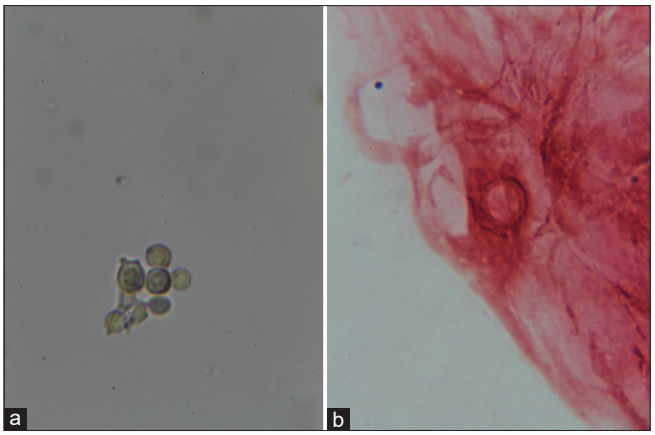

Figure 2: Microscopic features of Acanthamoeba cyst in corneal scraping — (a) KOH and (b) Gram's stain. Note: Double-walled spherical cysts

the lesion resolved completely with scarring with vision of 20/60 which further improved on contact lens trial to 20/30 [Fig. 1d]. The patient was continued on eye drops PHMB (0.02%) 4 times a day and tear supplements for the next 4 weeks.

Discussion

Acanthamoeba are ubiquitous microorganisms which cause a variety of systemic infections as well as keratitis.[7] FromIndia, Sharma *et al*. published the first case report of *Acanthamoeba* keratitis.[8,9] Several reports and updates on *Acanthamoeba* keratitis have been published from all over the world over time.[10-12] In developing countries such as India, trauma is the most common predisposing factor as opposed to contact lens wear reported in western literature.[7] In the life cycle of *Acanthamoeba*, trophozoites are sensitive to most available chemotherapeutic agents. However, presence of *Acanthamoeba* cysts in the body leads to persistent infection, against which very few agents are effective.[13] Biguanides and diamidines have been reported to have the best cysticidal activity.[14,15] Based on vastly reported literature, topical polyhexamethylene biguanide (PHMB 0.02%) remains the primary therapy.[16] Adjuvant therapy with chlorhexidine 0.02% has shown enhanced resolution of *Acanthamoeba* keratitis.[15,17] This case did not show improvement with PHMB monotherapy; however, when chlorhexidine was added as adjuvant therapy, the keratitis resolved. This emphasizes the treatment of *Acanthamoeba* keratitis with combination therapy as opposed to monotherapy.

With the advent of effective antiamoebic therapy surgical intervention for *Acanthamoeba* keratitis has been on a decline and reserved only for cases threatening limbus, perforations, and those worsening on medical therapy.[12] Moreover graft failure and recurrence of infection in grafts are known complications after surgery.[18] This patient was successfully managed on medical therapy alone, thus circumventing the need for a keratoplasty, which in children is always a challenge.

Several species of *Acanthamoeba* have been identified which are broadly designated in three groups based on their cyst morphology. However, on the basis of their nuclear gene (18S rDNA) sequences, *Acanthamoeba* species are recognized as at least 20 genotypes (T1-T20).[19] Short (<500 bp) 18S rDNA fragments have been able to identify the different strains. The

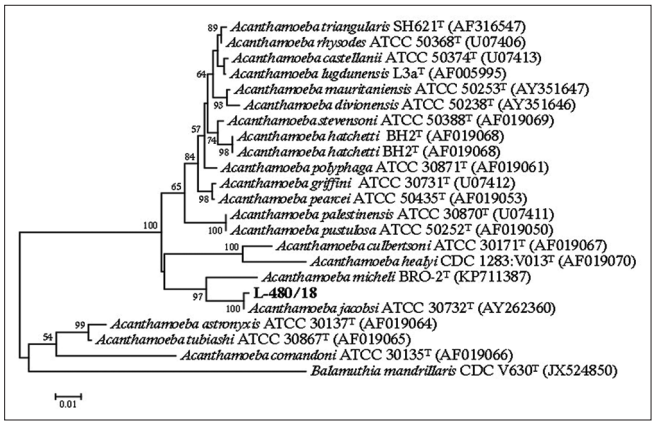

Figure 3: Neighbor-joining distance tree based on partial 18S rDNA sequences showing the clustering of clinical isolate L-480/18 with its closest relative *Acanthamoeba jacobsi* (shown in bold). Bootstra *p* values (1,000 replicates) of 50% are given at the nodes. *Balamuthiamandrillaris* was used as an outgroup. The 18S rDNA sequences of all the type strains were retrieved from NCBI database. Scale bar represents the number of substitutions per nucleotide position

specificgenotype T15, corresponds to *Acanthamoeba jacobsi*. While studies from India and worldwide report T4 as the most prevalent genotype in AK patients, keratitis due to T15 strain, to the best of our knowledge has not been reported before.[20-22]

Behera *et al*. from India, in their phylogenetic analysis reported 16 of the 20 isolates to be of T4, two of genotype T10 and the remaining two isolates of unassigned genotypes.[20] T4 was also the most commonly isolated strain in studies by Derda *et al*., Parischa *et al*., and Ertabaklar *et al*.[21-23] Additionally, the cysts of *Acanthamoeba jacobsi* bear a striking resemblance to the cysts of *Dictyostelium polycephalum* that has been reported by Reddy *et al*.[5] There are very few reported cases of the same and the management of such cases remains a dilemma.

Prognosis of *Acanthamoeba* keratitis depends on correct timely diagnosis and appropriate treatment. Advances in microbiological techniques further help in identification of the organism as well as the species. In this case, gene sequencing not only helped us to distinguish *Acanthamoeba* species from *Dictyostelium* species but also helped to identify the specific genotype (T15).

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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