



Assessment of pathogenic potential of *Acanthamoeba* isolates by in vitro and in vivo tests

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Abstract

Acanthamoeba are free-living protozoa present ubiquitously in numerous environmental reservoirs that exist as an actively feeding trophozoite or a dormant cyst stage. The pathogenic *Acanthamoeba* are known to cause *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE). Despite their omnipresence, the number of infections is quite low. The reason behind this low frequency of *Acanthamoeba* infections could be the existence of many non-pathogenic strains or a successful host immune response to these infections. Studies in the past have proposed a few physiological parameters for the differentiation of pathogenic and non-pathogenic strains. Additionally, in vivo experiments are known to play an essential role in understanding the virulence of parasites, immunological aspects, and disease pathogenesis. The thermotolerance (30 °C, 37 °C, and 40 °C) and osmotolerance (0.5 M, 1 M, and 1.5 M) tests were performed on 43 *Acanthamoeba* isolates from patients with keratitis ($n=22$), encephalitis ($n=5$), and water samples ($n=16$). In addition, the genotype of 10 *Acanthamoeba* isolates (keratitis ($n=2$), encephalitis ($n=2$), water ($n=6$)) was determined and were then evaluated for pathogenicity on mouse model by inducing *Acanthamoeba* keratitis and amoebic encephalitis. The results of the thermotolerance and osmotolerance assays categorized 29/43 (67.4%) isolates as pathogenic, 8 as low pathogenic (18.6%), and the remaining 6 (13.9%) as non-pathogenic. The 10 *Acanthamoeba* isolates were categorized as T11 (5 isolates), T5 (2 isolates), T4 (2 isolates), and T10 (1 isolate) genotypes. Out of 10 *Acanthamoeba* isolates, 9 were successful in establishing AK, amoebic encephalitis, or both in the mice model, and a single isolate was found non-pathogenic. Two isolates from water samples were non-pathogenic in the physiological tests but successfully established *Acanthamoeba* infection in the mice model. The results of the physiological assays and in vivo experiments were analogous for 7 isolates while 1 isolate from the water was low pathogenic in the physiological assays but failed to produce pathogenicity during in vivo experiments. The physiological parameters are not very dependable to test the pathogenic potential of *Acanthamoeba* isolates, and thus results must always be validated by in vivo experiments. There is no infallible approach for determining the potential pathogenicity of environmental isolates of *Acanthamoeba* because several parameters regulate the pathogenic potential.

Keywords *Acanthamoeba* keratitis · Granulomatous amoebic encephalitis · In vivo model · Osmotolerant · Physiological assay · Thermotolerant

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Introduction

Acanthamoeba, a genus of single-celled protozoa, encompasses a diverse group of microorganisms that thrive in various aquatic and terrestrial environments. These organisms exhibit intricate biological characteristics and play significant roles in ecosystems yet they also pose a potential threat to human health (Garcia et al. 2013; Ong et al. 2017). Upon successful invasion of the human host, the pathogenic isolates are responsible for causing serious illness of the central nervous system (granulomatous amoebic encephalitis-GAE), eye (*Acanthamoeba* keratitis-AK), and rarely cutaneous or disseminated disease (Siddiqui and Khan 2012; Khurana and Sharma 2020; Lorenzo-Morales et al. 2015). GAE is mainly documented in immunocompromised patients and is associated with high mortality (da Rocha-Azevedo et al. 2009). AK is mainly reported among individuals wearing contact lenses, or those sustaining injuries to the eyes, and loss of vision is a common outcome (Dodangeh et al. 2018; Khan 2009; Megha et al. 2021). Also, various serological surveys have indicated the presence of *Acanthamoeba*-specific serum antibodies in about 90% of the healthy adult population but infections are relatively rare which may be attributed to an effective host response or the presence of non-pathogenic strains (Alizadeh et al. 2001; Niederkorn 2021). Hence it is crucial to study the factors and triggers related to pathogenicity and thus it is essential to differentiate reliably between pathogenic and non-pathogenic isolates. Many studies have suggested physiological parameters for determining pathogenicity, but they are not very precise and reliable (Dodangeh et al. 2018; Khan and Tareen 2003). With this background, we have attempted to check the reliability of thermo-tolerance, osmotolerance assays, and in vivo studies for determining the pathogenic potential of *Acanthamoeba* isolates.

Materials & Methods

Sampling, and confirmation of *Acanthamoeba*, and maintenance of *Acanthamoeba*

The study included 43 isolates obtained from patients with keratitis ($n = 22$), encephalitis ($n = 5$), and water samples ($n = 16$). The isolates CHA1 to CHA27, CHA29, and CHA30 have been published previously (Khurana et al. 2015; Megha et al. 2018, Megha et al. 2021; Megha et al. 2023). The isolates CHA32-CHA46 were recorded for the first time in the present study, however, genotyping was not performed for all the isolates. The keratitis and

encephalitis isolates were obtained from clinical specimens (corneal scraping, cerebrospinal fluid, brain biopsy) and inanimate samples (contact lens and lens solution) of patients who had presented to the Postgraduate Institute of Medical Education and Research, Chandigarh, India. The water samples were obtained from different locations in Chandigarh, India, and are being maintained in the Department of Medical Parasitology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. The water sample was centrifuged, and the pellet was inoculated onto the non-nutrient agar (NNA) plate having a lawn of *Escherichia coli*, and the growth of *Acanthamoeba* was monitored until seven days.

Physiological parameters for discriminating pathogenic and non-pathogenic *Acanthamoeba*

The temperature and osmolarity tolerance assays were performed on 43 isolates. For the thermo-tolerance assay, an agar block containing *Acanthamoeba* cysts was sliced, kept on the surface of a freshly prepared 1.5% non-nutrient agar (NNA) agar plate having a lawn of *Escherichia coli*, and incubated in triplicates at three different temperatures; 30 °C, 37 °C, and 40 °C. For the osmolarity tolerance assay, an agar block containing *Acanthamoeba* cysts was sliced and kept on the surface of freshly prepared 1.5% NNA agar plates having different mannitol (D-Mannitol, HiMedia Pvt. Ltd., New Delhi, India) concentrations (0.5 M, 1 M, and 1.5 M) and having a lawn of *E. coli* that were incubated at 30 °C. The growth in the plates was monitored at 24, 48, and 72 h for their ability to grow. The limit of expansion was scored based on the distance reached by the trophozoites and cysts from the center of the plate as described previously (Khan et al. 2001; Mohd Hussain et al. 2022a; Possamai et al. 2018). The strains showing maximum growth (+ + +) at the high temperature (40 °C) and mannitol concentration (1.5 M) were designated pathogenic whereas the non-pathogenic strains were the ones incapable of proliferating at high temperature (40 °C) and mannitol concentration (1.5 M). The strains exhibiting scanty growth (+) at high temperature (40 °C) and mannitol concentration (1.5 M) were designated low-pathogenic (Possamai et al. 2018).

Animal model for the differentiation of pathogenic and non-pathogenic *Acanthamoeba*

We evaluated 10 *Acanthamoeba* isolates (2 isolates each of keratitis, encephalitis, and 6 isolates from water) in the mouse model for the establishment of keratitis and encephalitis. A total of 120 male Balb/c mice were used in the study as detailed below. All the animal experimentation was done according to the guidelines of the Committee for Control and

Supervision of Experiments on Animals and recommendations of the Institutional Scientific Advisory Committee. All the necessary approvals were taken from the Institutional Ethics Committee (Ref. No. 95/93/IAEC/658).

Maintenance of *Acanthamoeba* isolates

All the *Acanthamoeba* isolates were inoculated on non-nutrient agar (NNA) having a lawn of inactivated *E. coli* and incubated at 37 °C for 72 h. The trophozoites were scraped, and suspended in sterile phosphate-buffered saline (PBS); centrifuged at a low speed and the pellet was washed with 0.9% isotonic NaCl, then the pellet was resuspended in PBS and the cell count was adjusted to 10^3 trophozoites/ml (Sharma et al. 2021).

Acanthamoeba keratitis

The pathogenic potential of ten *Acanthamoeba* isolates was evaluated using a keratitis model on 40 mice (4 mice per isolate). The right eyes of all the mice were used for infection, and the left eyes were used as control. The mice were given 0.1 ml of an anaesthetic cocktail per 20 g of body weight (ketamine 60 mg/kg + xylazine 5 mg/kg), and 0.5% proparacaine ophthalmic solution was also used to achieve corneal anaesthesia. The eyes were traumatized using a surgical blade no. 15 three times vertically and horizontally under a dissection microscope. Small lenses made of Parafilm that had been preincubated with *Acanthamoeba* were then applied to the abraded corneal surface. The eyelids were sewn by performing tarsorrhaphy using 6–0 (0.7 metric) vicryl sutures as described previously (Sharma et al. 2021). The mice were graded on the fifth-day post-infection based on the clinical features of mild to severe corneal cloudiness & presence of corneal infiltrate. The mice presenting with clinical features were euthanized on day seven, and the eyes were removed for culture, stored in PBS for *Acanthamoeba*-specific PCR (Schroeder et al. 2001), and stored in formalin for histopathological examination.

Amoebic encephalitis

The amoebic encephalitis mouse model was performed on 80 Balb/c mice (8 mice per isolate including 5 mice for infection and 3 as controls) with 10 *Acanthamoeba* isolates. Amoebic encephalitis was established with slight modifications in the protocol previously described (Mirjalali et al. 2013; Omaña-Molina et al. 2017). Briefly, the mice were immune-suppressed with 2 doses of cyclophosphamide (EndoxanTM-N, Cadila Healthcare (Zydus), Ahmedabad, India) injected intra-peritoneally at a dosage of 100 mg/kg on alternate days. The immune-suppressed mice were anesthetized with 0.1 ml of anesthetic cocktail (ketamine 60 mg/kg + xylazine 5 mg/

kg) for an easy intranasal instillation of 10^3 *Acanthamoeba* trophozoites. The mice were monitored daily for signs of encephalitis like in-coordination, ruffled hair, and lethargy for 21 days. The mice presenting with severe clinical features were sacrificed earlier and those healthy were sacrificed at the end of the time-point (21 days). The brain, lung, liver, and spleen were removed from euthanized mice and used for culture, kept in PBS for PCR (Schroeder et al. 2001), and stored in formalin for histopathologic examination.

Sequencing for genotype determination

The genotyping for isolates CHA5, CHA20, CHA24, and CHA 27 had been performed previously and the accession numbers are provided in Table 2. The purified amplified products of the remaining six isolates were sequenced using the Big-Dye 3.1 Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130 Genetic Analyzer automated sequencer following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The obtained raw sequences were compared with GenBank by using Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for determining the genotype and the sequence similarity.

Results

The thermo-tolerance and osmo-tolerance assays were performed on 43 *Acanthamoeba* isolates including 22 isolates from AK, 5 from GAE patients, and 16 from water samples. All the isolates were previously inoculated on an NNA medium and confirmed by PCR assay for their confirmation as *Acanthamoeba* species. Twenty-nine out of 43 isolates (67.4%) showed pathogenic potential based on thermo-tolerance and osmo-tolerance assays, 8 were low pathogenic (18.6%), and the remaining 6 (13.9%) isolates were not able to tolerate high temperature and osmolarity conditions and thus were designated non-pathogenic (Table 1). In addition to the physiological assays, 10 isolates were sequenced for determining the specific genotype and were subsequently employed in AK and amoebic encephalitis animal models each to assess the pathogenic potential of the *Acanthamoeba* isolates. In the present study, ten *Acanthamoeba* sequences that belonged to T4, T5, T10, and T11 genotypes were detected upon comparing the raw data to the NCBI database. The genotype T10 was detected in one clinical isolate and T5 was found in two isolates from water. Furthermore, five isolates were similar to the T11 genotype and one isolate each from a keratitis patient and water was detected as the T4 genotype (Table 2). The “Identity to reference accession number” in Table 2 represents the accession number to which our isolate showed the highest similarity.

Table 1 Results of the thermotolerance and osmotolerance assay for the *Acanthamoeba* isolates at 72 h incubation

Isolate No	Source	30 °C	37 °C	40 °C	0.5 M	1 M	1.5 M	Predicted Pathogenicity
CHA1	Keratitis	+++	+++	+++	+++	+++	++	Pathogenic
CHA2	GAE	+++	++	+	+++	+++	+	Low pathogenic
CHA3	Keratitis	+++	+++	+++	+++	+++	++	Pathogenic
CHA4	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA5	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA6	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA7	Water	+++	++	+	+++	++	+	Low pathogenic
CHA8	GAE	+++	++	+	+++	++	++	Pathogenic
CHA9	Water	+++	+++	+++	+++	+++	+++	Pathogenic
CHA10	Water	+++	+++	+++	+++	+++	+++	Pathogenic
CHA11	Keratitis	+++	+++	+++	+++	+++	++	Pathogenic
CHA12	Keratitis	+++	+++	+++	+++	+++	++	Pathogenic
CHA13	Keratitis	+++	+++	+++	+++	++	++	Pathogenic
CHA14	GAE	+++	+++	+++	+++	+++	++	Pathogenic
CHA15	Keratitis	+++	+++	+++	+++	+++	++	Pathogenic
CHA16	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA17	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA18	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA19	Keratitis	+++	+++	+	+++	+++	+	Low pathogenic
CHA20	Keratitis	+++	+++	+	+++	+++	++	Pathogenic
CHA21	Keratitis	+++	+++	+++	+++	+++	++	Pathogenic
CHA22	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA23	Keratitis	+++	+++	+	+++	+++	+	Low pathogenic
CHA24	GAE	+++	+++	++	+++	+++	++	Pathogenic
CHA25	Keratitis	+++	+++	++	+++	+++	++	Pathogenic
CHA27	Brain abscess	+++	+++	++	+++	+++	++	Pathogenic
CHA29	Water	+++	+++	+++	+++	+++	+++	Pathogenic
CHA30	Water	+++	+++	++	+++	+++	++	Pathogenic
CHA32	Keratitis	+++	+++	+	+++	+++	+	Pathogenic
CHA33	Keratitis	+++	++	-	+++	++	-	Non pathogenic
CHA34	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA35	Keratitis	+++	+++	+++	+++	+++	+++	Pathogenic
CHA36	Water	+++	++	+	+++	++	+	Low pathogenic
CHA37	Water	+++	+	-	+++	+	-	Non-pathogenic
CHA38	Water	++	++	+	++	++	+	Low pathogenic
CHA39	Water	+++	++	+	++	++	+	Low pathogenic
CHA40	Water	+++	++	++	+++	++	++	Pathogenic
CHA41	Water	++	+	-	-	-	-	Non-pathogenic
CHA42	Water	+	++	++	+	++	++	Pathogenic
CHA43	Water	+++	+++	-	+	+	-	Non-pathogenic
CHA44	Water	+++	+++	-	+	-	-	Non-pathogenic
CHA45	Water	+++	+++	+	++	++	+	Low pathogenic
CHA46	Water	+++	+++	-	+++	-	-	Non-pathogenic

No growth (-), Growth until 1 cm from the central area (+), Growth from 1 to 2 cm from the central area (++), and Growth above 2 cm from the central area (+++); GAE-Granulomatous amoebic encephalitis

Pathogenic: Maximum growth (+++) at the high temperature (40 °C) and mannitol concentration (1.5 M)

Non-pathogenic strains: No proliferation at high temperature (40 °C) and mannitol concentration (1.5 M)

Low-pathogenic: Scanty or retarded proliferation (+) at high temperature (40 °C) and mannitol concentration (1.5 M)

Table 2 Genotype of the sequences obtained in the present study and the corresponding identity to reference accession number

Isolate	Source	Genotype	Accession number	Identity to reference accession number
CHA5	Keratitis	T11	KX709491.1	KJ094683
CHA20	Keratitis	T4	KY859173.1	KF733234
CHA24	CSF	T11	KX675341.1	KU936113
CHA27	Brain abscess	T10	KX675337.1	AF019067
CHA36	Water	T4	Unpublished	OQ300084.1
CHA37	Water	T11		KJ094683.1
CHA39	Water	T5		KM823783.1
CHA40	Water	T11		AF333608.1
CHA45	Water	T11		KJ094683.1
CHA43	Water	T5		MN153019.1

Table 3 Results of the physiological assays and in vivo *Acanthamoeba* keratitis and amoebic encephalitis mouse model for ten *Acanthamoeba* isolate

Isolate	Source	Result of Physiological parameters	Genotype	Result of in vivo experimentation			
				<i>Acanthamoeba</i> keratitis		No. of animals positive (GAE)	
				No. of animals positive	Interpretation	No. of animals positive	Interpretation
CHA5	Keratitis	Pathogenic	T11	4/4	Pathogenic	Negative	Non-pathogenic
CHA20	Keratitis	Pathogenic	T4	3/4	Pathogenic	Negative	Non-pathogenic
CHA24	CSF	Pathogenic	T11	2/4	Pathogenic	6/6	Pathogenic
CHA27	Brain abscess	Pathogenic	T10	3/4	Pathogenic	5/6	Pathogenic
CHA36	Water	Low pathogenic	T4	Negative	Non-pathogenic	Negative	Non-pathogenic
CHA37	Water	Non-pathogenic	T5	4/4	Pathogenic	3/6	Pathogenic
CHA39	Water	Low pathogenic	T5	3/4	Pathogenic	Negative	Non-pathogenic
CHA40	Water	Pathogenic	T11	4/4	Pathogenic	Negative	Non-pathogenic
CHA45	Water	Low pathogenic	T11	4/4	Pathogenic	Negative	Non-pathogenic
CHA43	Water	Non-pathogenic	T11	4/4	Pathogenic	Negative	Non-pathogenic

Forty mice were used to investigate the pathogenic potential of 10 isolates to cause keratitis, and the clinical assessment was carried out 5 days after infection. The 2 AK isolates, 2 encephalitis, and 5 environmental isolates successfully established AK in the mice model (Table 3). The successful establishment of AK in the mice eye was confirmed clinically by the presence of characteristic corneal infiltrate and corneal cloudiness. In addition, the inoculation of corneal scraping demonstrated *Acanthamoeba* trophozoites and cysts on culture (Fig. 1) and was used for the successful amplification of the *Acanthamoeba* by PCR (Fig. 2). The clinical and histopathological examination of the mouse cornea further validated AK in the mouse model (Fig. 3). The three confirmatory tests corroborated with the clinical evaluation of the mouse eye. In addition to the AK

model, we have assessed the pathogenic potential of 10 *Acanthamoeba* isolates in an amoebic encephalitis mice model. Out of 10 isolates, the 2 GAE, and 1 environmental isolate successfully established an amoebic encephalitis model that was confirmed using culture (Fig. 1), PCR (Fig. 2), and histopathology of the brain, lung, liver, and spleen (Fig. 4) from the infected mice. Based on the results of physiological parameters and in vivo experiments, the 4 clinical isolates were designated pathogenic. In addition, 4 isolates from the water had the potential of inducing keratitis alone, and 1 isolate was capable of inducing keratitis and encephalitis in the animal model. The result of the physiological assays for these 5 isolates was quite variable that designated 1 isolate as pathogenic; 2 isolates each as low pathogenic and non-pathogenic (Table 3). Additionally, 1 isolate

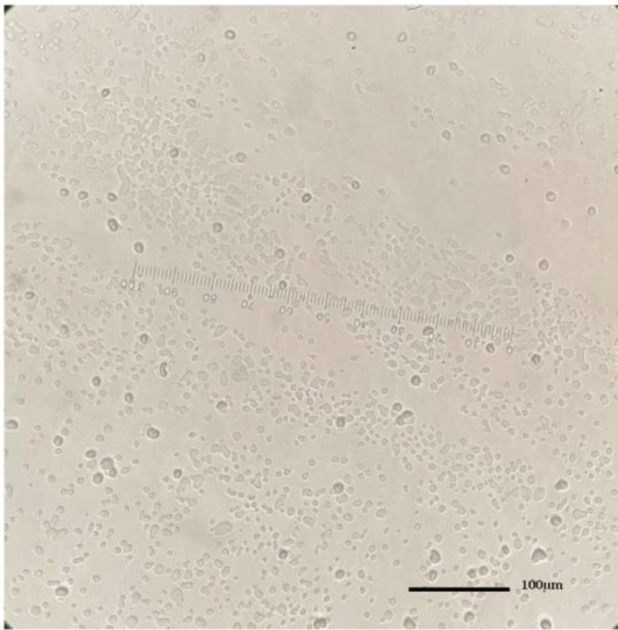


Fig. 1 *Acanthamoeba* trophozoites and cysts on 1.5% non-nutrient agar (10× magnification)

was found non-pathogenic in the in vivo experimentation and it was designated as low pathogenic by the physiological assays.

Discussion

Acanthamoeba species are ubiquitous in nature; however, cases of AK and GAE have been relatively rare (Lorenzo-Morales et al. 2006; Rezaeian et al. 2008; Possamai et al. 2018). Many different parameters in the past have helped in demarcating pathogenic isolates from non-pathogenic *Acanthamoeba*. Studies have used molecular and protease markers, in vivo models, and physiological parameters to assess the pathogenic potential of *Acanthamoeba* isolates (Howe et al. 1997; Khan et al. 2000; Khan et al. 2001; Khan et al. 2002; Garate et al. 2006; Castro-Artavia et al. 2017). Additionally, comparisons between osmotolerance, thermotolerance, and *Acanthamoeba* genotypes have been reported previously (Kahraman & Polat 2022). The virulence of an isolate depends on its capability to adapt & survive in the mammalian host and the isolates taken from environmental sources should be evaluated by animal experimentation for their pathogenicity (Khan 2006; Landell et al. 2013; Kahraman & Polat 2022). In addition, studies in the past have shown that the pathogenic potential of free-living amoebae, including those from the genus *Acanthamoeba*, may be

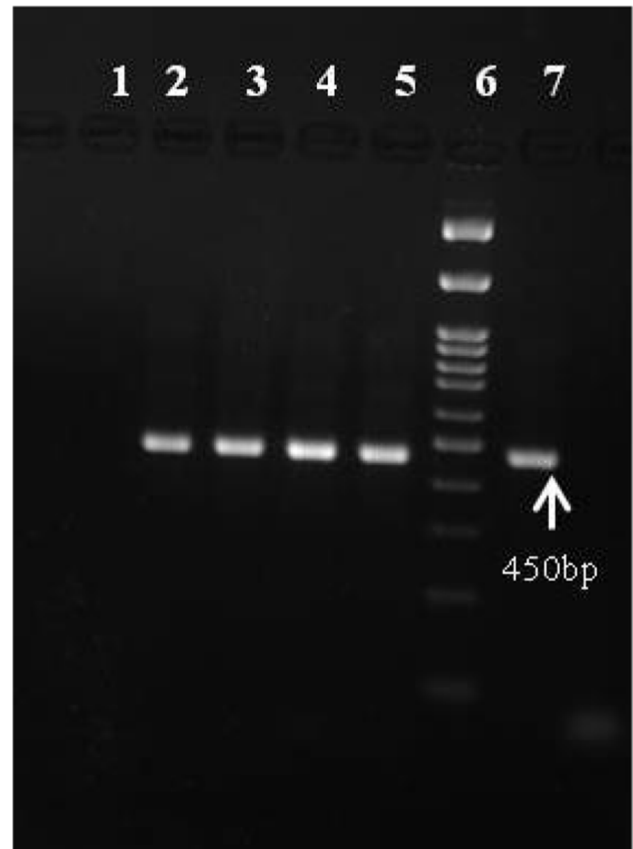


Fig. 2 Representative agarose gel image for *Acanthamoeba* spp.-specific JDP PCR, lane 1, negative control, lane 2 & 3, positive samples of corneal scraping, lane 4 & 5, samples from mouse brain & lung found positive, lane 6, 100 bp molecular marker, lane 7, positive control amplifying a ~450 bp band

examined in vitro using known cell lines or primary and secondary human cell cultures. Additionally, using them under controlled conditions reduces the individual and species-specific variability of the experimental animals, enhances the reproducibility of the experiment, and provides excellent data on both the pathogenicity characteristics of these protozoa and the defensive response of the host (Koehsler et al. 2009; Martín-Navarro et al. 2010; Mohd Hussain et al. 2022b; Walochnik et al. 2000). The pathogenic *Acanthamoeba* is reported to withstand high temperatures and osmolarity and also show high levels of heat shock proteins (Pérez-Serrano et al. 2000; Podlipaeva et al. 2006; Landell et al. 2013; Kahraman & Polat 2022). In the present study, we have used physiological parameters of thermo-tolerance and osmo-tolerance along with results from in vivo study to ascertain the pathogenicity of isolates. The verification of isolates based on the results of the animal model has helped in providing an appropriate confirmation. Previous reports

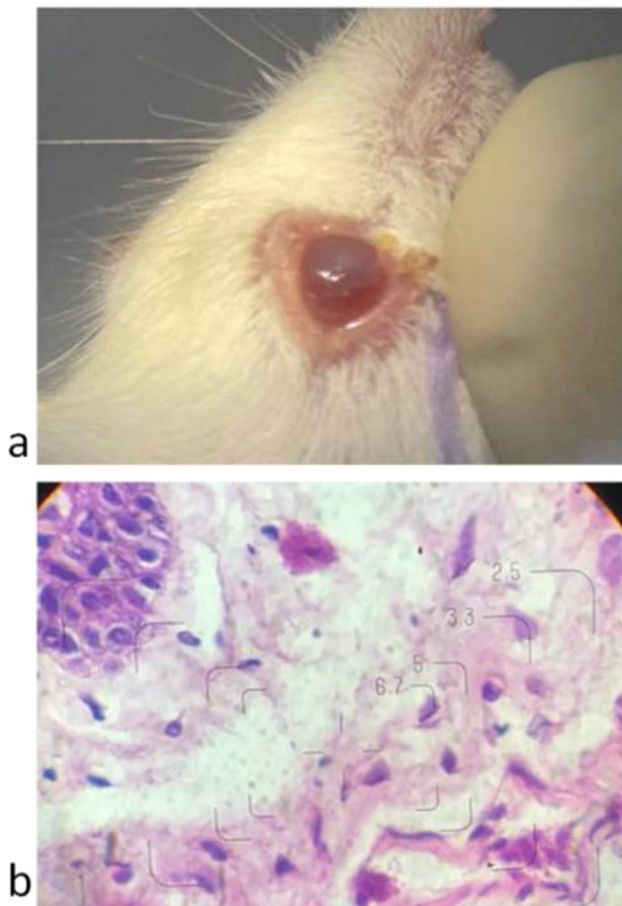


Fig. 3 Clinical and histopathological examination of *Acanthamoeba* infected mouse eye. **a** Corneal cloudiness and infiltrate seen at day five post-infection; **b** Hematoxylin and eosin stained section displaying *Acanthamoeba* trophozoites in the substantia propria (40X magnification)

have assessed the pathogenicity based on different physiological parameters and cytopathogenicity that suggested a weak correlation between the clinical origin of isolate and physiological assays (Nagyova et al. 2010; Siddiqui and Khan 2012; Possamai et al. 2018), but they did not perform an animal model based test to confirm their results from in vitro experiments.

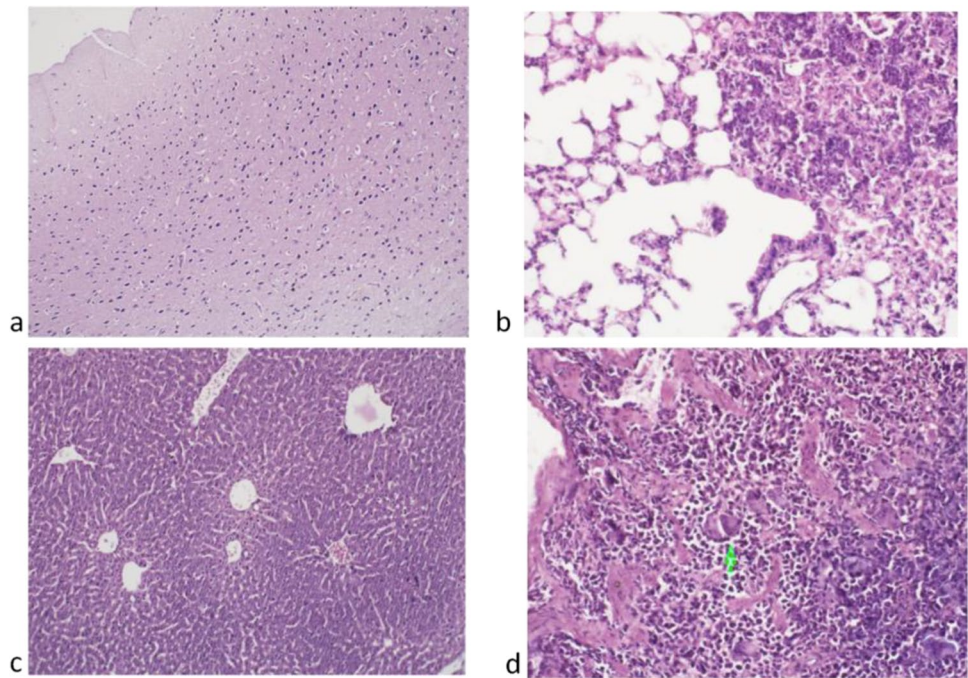
The physiological criteria in the current study misclassified 4 clinical isolates either as low or non-pathogenic. Three out of 27 clinical isolates were detected as low pathogenic and 1 was non-pathogenic. Among 16 isolates from water sources, physiological assays suggested 6 isolates as pathogenic and the remaining 10 were either low pathogenic or non-pathogenic (Table 1). Additionally, based on genotyping, the 10 clinical and environmental isolates included for the in vivo experimentation were categorized

into genotypes T4, T5, T10, and T11 (Table 2). Studies in the recent past have demonstrated the presence of T4, T5, and T11 genotypes in samples from water, soil, and dust (Behnia et al. 2017; Milanez et al. 2020; Paknejad et al. 2020; Salehi et al. 2022). Likewise, different *Acanthamoeba* genotypes have been associated with clinical cases of keratitis and encephalitis with T4 being the most common genotype among all (Esboei et al. 2020; Hajjalilo et al. 2016; Kalra et al. 2020; Megha et al. 2018). The fact that we did not genotype every isolate makes it unjust to say which genotype is the most prevalent.

The ten isolates used for in vivo experiments included environmental and clinical isolates each of keratitis and encephalitis. The keratitis isolates (CHA 5 and CHA 20) were obtained from the corneal scrapings of AK patients. At the time of presentation, the size of the ulcers was 1×1 mm and 0.5×0.5 mm respectively and the visual acuity was 6/6. The patients were prescribed Polyhexamethylene Biguanide (PHMB 0.02%) half hourly for one week, then hourly for one week and then gradually tapered according to the response. The clinical outcomes in both cases were healed corneal opacities. On the other hand, the two encephalitis isolates belonged to an immunocompetent individual (CHA24) and an individual under treatment for acute myeloid leukaemia (CHA27). The diagnosis of GAE was made postmortem in the case of CHA24 and antemortem for CHA27 isolates.

Based on the results of in vivo mouse model, 5 environmental isolates were found to establish keratitis, amoebic encephalitis, or both, and 1 was found non-pathogenic (Fig. 1–4). We could not conduct in vivo experiments on a larger set of *Acanthamoeba* isolates from water due to certain constraints in procuring a sufficient number of animals. The results of the physiological assays and in vivo experiments were analogous for 7 isolates. In addition to the establishment of AK and amoebic encephalitis by the respective clinical isolates, we observed the GAE isolate establish keratitis as well. Our results are different from a previous report in which the isolate from the keratitis patient was not showing positive results in the keratitis mouse model. However, there was a direct relationship between their physiological results and animal experiments (Mirjalali et al. 2013). The long-term axenic cultivation of the strain and certain host factors might have played a role that led to this discordance. The growth at high osmolarity has been correlated with the propensity to counter high osmotic pressure, a situation *Acanthamoeba* face while acting as parasites of the cornea (Siddiqui and Khan 2012). Additionally, the capacity of thermo-tolerance is directly proportional to the ability of *Acanthamoeba* to produce cellular damage (Walochnik et al. 2000). Even if these two characteristics provide

Fig. 4 Histopathological examination of hematoxylin and eosin stained sections of brain, lung, liver, and spleen of mouse infected with amoebic encephalitis. **a:** Brain section displaying hypoxia in the form of red shrunken neurons; **b:** Alveolar spaces in the lung studded with neutrophils & macrophages, congestion and necrotizing pneumonia can be seen; **c:** Sinusoidal congestion and portal vein dilation observed in liver; **d:** Spleen section displaying mild hyperplasia (10X magnification)



amoebae with an adaptative advantage while parasitizing a host, the mere presence or absence of these characteristics is not enough to define the amoeba as pathogenic. For instance, different species of *Acanthamoeba* may be thermotolerant and osmotolerant but they are not always pathogenic (De Jonckheere 1980; Schuster and Visvesvara 2004). In addition, the pathogenic potential of environmental isolates of *Acanthamoeba* cannot be determined with absolute certainty because other factors, such as the genetic makeup of the protozoan, epigenetic factors, and the biological characteristics of the experimental models (animal, tissue, or cellular) must also be taken into account. In our data, none of the pathogenic isolates from the physiological assay was found non-pathogenic in the mouse model. However, this could be explained by the less number of isolates included in our study for in vivo experimentation. Nevertheless, physiological properties are not sufficient to ascertain whether a given isolate is always pathogenic. There are other aspects like the ability to invade the host, protease profile, and encystment rate which play a role in the pathogenesis and might act as an indicator of pathogenicity.

Author's contributions The study conception, funding acquisition, review and editing, project administration, supervision was done by Sumeeta Khurana. Chayan Sharma performed the experimental work and data analyses. Chayan Sharma and Kirti Megha prepared the figures and formal analysis. Anchal Thakur and Amit Gupta performed the clinical evaluation of the mouse model. Alka Bhatia performed the histopathological analysis. All the authors have read and approved the final manuscript.

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Data availability All the necessary data has been added to the main manuscript file.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval Institutional Review Board Statement: The work was duly approved by the Institutional Ethics Committee vide NK/5283/PhD/213 dated 28–03-2019 and the Institutional Animal Ethics Committee vide 95/93/IAEC/658 dated 13–10-2018.

Consent to participate Not applicable.

Conflicts of Interest/Competing Interests The authors declare no conflict of interest.

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