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Original Article

Pathogenic Assays of *Acanthamoeba* Belonging to the T4 Genotype

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Abstract

Background: *Acanthamoeba* genus is introduced as opportunistic and cosmopolitan parasite. Monkey and wistar rat are appropriate models for experimental study on *Acanthamoeba* infection. In this study *Acanthamoeba* spp. were isolated from hot spring (HS), windows dust (WD) and a corneal sample of keratitis patient (KP) and their pathogenicity surveyed by in vitro and in vivo tests.

Methods: Isolates of *Acanthamoeba* were cultivated axenically for 12 months in PYG medium. Overall, 30 wistar rat**s,** in 6 equal groups were used for developing experimental *Acanthamoeba keratitis* (AK) and Granulomatous Amoebic Encephalitis (GAE). The Keratitis and Granulomatous Encephalitis experiments were performed by intrastromal and intranasal inoculation of *Acanthamoeba* cysts, respectively. Pathogenicity of the three isolates was also evaluated by in vitro test using osmotolerance and temperature tolerance assays. Identification of genotypes were performed by PCR technique and sequencing.

Result: None of the isolates could perform AK and GAE in wistar rats, although all isolates were described as T4 genotype. Isolates obtained from KP and WD could grow only in 30 °C, but not in 37 °C and 40 °C. On the other hand, HS isolate grew in 30 °C and 37 °C but not in 40 °C. Moreover, all of isolate grew in 0.5 M mannitol but not in 1 M and 1.5 M.

Conclusion: T4 isolates with a long-term axenic culture and different factors related to host and parasite may play role in pathogenicity of these free-living amoebae.

Introduction

Acanthamoeba genus was detected for the first time in *Cryptococcus* culture and was described by volkonsky in 1931(1, 2). These free-living protozoa are an opportunistic pathogen of human beings with worldwide distribution. These amoebae has been reported from different sources such as soil, freshwater, dust, air, contact lenses, dialyze unit, ventilation system and distilled water bottles and dead animals (2-4).

The life cycle of *Acanthamoeba* includes two stages: dormant cyst (8-30 µm) and motile trophozoite (8-40µm). Trophozoite can feed on small organisms and reproduce by binary fission in optimal conditions. *Acanthamoeba* encyst into a resistant double-layer wall under harsh conditions (2, 5, 6). This genus derivates to two strains including non-pathogen and pathogen cases (3). Pathogenic strains can be causative agents of amoebic keratitis (AK) in healthy persons who are contact lens wearer or have a history of corneal trauma. On the other hand, Granulomatous Amoebic Encephalitis (GAE), a fatal infectious disease, and skin sore could be observed in immune-compromised patients (3, 6-8). *Acanthamoeba* encephalitis is rare and is less likely to be diagnosed with successful medical intervention (2).

Several genotypes of *Acanthamoeba* (T1-T17) have been identified (9-11) so far. However; most cases of keratitis and non-keratitis infections in human are due to T4 genotype (2, 9). In Iran T4 genotype described from different sources and most of *Acanthamoeba* related keratitis was attributed to T4 type (12). To study pathogenicity of *Acanthamoeba*, several line of animal models have been employed. Larkin and Easty studied *Acanthamoeba* keratitis on wistar rat (13), Ren et al. evaluated experimental keratitis on wistar rat as well (14). In some studies pig, rabbit and mice also used as animal model for experimental *Acanthamoeba* infection (15). In addition, in vitro tests such as osmotolernace and temperature tolerance assays are proved as an evaluation tests for pathogenicity of *Acanthamoeba* isolates (16-18).

In this study, the axenificatiion of *Acanthamoeba* spp. were done and the pathogenicity of *Acanthamoeba* T4 genotypes from different sources were surveyed using in vitro (temperature tolerance and osmotolerant tests) and in vivo test (animal- based study).

Material and Methods

Acanthamoeba isolates and culture

Hot Spring (HS) and window dust (WD) isolates were obtained from our previous studies (12, 19). Dust and hot spring strains were cultivated in Bactoagar medium 1.5%, enriched with *Escherichia coli* (non-nutrient agar medium). The clinical sample isolated from a 17-year old man affected by AK, with a history of soft contact lens use. The clinical manifestation includes, unilateral decreased visual acuity, sensitivity to light, redness, foreign body sensation, tearing and eye pain.

Axenic culture

All three isolates were axenically cultured in protease pepton, yeast extract and glucose (PYG) medium (0.75%, w/v, proteose peptone; 0.75%, w/v, yeast extract; 1.5%, w/v, glucose) in T-25 tissue-culture flasks at 27°C. The Cysts were concentrated by centrifugation at 3500 rpm for 10 min and used for experimental tests.

DNA extraction, PCR and Sequencing

Acanthamoeba cysts were harvested from axenic medium by centrifuging in 3500 rpm for 5 min and were washed 3 times using normal saline. The genomic DNA of isolates was extracted by conventional Phenol- chlorophorm method. Polymerase chain reaction (PCR) was carried out by JDP1Primer (5'-GGCCCAGATCGTTTACCGTGAA-3') and JDP2 primer (5'-TCTCACAAGCTGCTA-GGGGAGTCA-3') which amplify a 500 fragme-nt of 18S rRNA gene. Each 50µl reaction mixture

contained 5µl of 10X buffer, 1.2U Taq polymerase (Fermentase, Thermo Fisher Scientific, Lithuania), 0.2mM of each dNTPs, 2mM $Mgcl_2$, 0.2 µM of each primers, and 3µl DNA sample. A negative control containing all reagents except DNA was included in all assays. Amplifications were performed in a TECHNE thermal cycler (UK) programmed for an initial heating at 95 °C for 1 min followed by 35 cycles at 94 °C for 35S, 56 °C for 45S, and 72 °C for 1 min, and a final extension of 72 °C for 5 min. PCR products electrophoresis was done on 2% agarose gel stained by ethidium bromide. All of isolates were sequenced by ABI 3130 (California, USA)and compared using BLAST GenBank database.

Pathogenicity test (in vitro and in vivo tests)

1. Osmotolerance Test

Mannitol test was performed on three isolates (HS, WD and KP). Bactoagar medium containing three concentration of Mannitol (1.5 M, 1M and 0.5 M) used for each isolate and growth of *Acanthamoeba* were evaluated at 24, 48 and 72 hours after culture by light microscopy examination (3, 17).

2. Temperature Tolerance Test

Acanthamoeba harvested from axenic culture was cultivated in Bactoagar plates and then each plate was placed in three different temperatures: 30 °C, 37 °C and 40 °C. Outgrowth of amoebae was monitored by light microscopy every 24 h for 3 days (16-18).

3. In vivo test using wistar rat

Thirty Wistar rats divided into 6 equal groups with same gender. Each group included 5 Wistar rat and the weight of each animal was about 130 gr.

Developing experimental Granulomatous Amoebic Encephalitis

Immune systems of three groups of rats were suppressed by met-hylprednizolone. Forty µg of met-hylprednizolone was injected intramuscular in two interval weeks (20 µg/weekly). After second week, approximately 1×10^6 cysts of each isolate inoculated intranasaly. After 4 weeks, 3 groups were anesthetized by chloroform and their brains transferred to the laboratory for tissue sections. Staining was performed by Haematoxylin and Eosin (H&E) and culturing was done in nutrient free agar medium.

Developing Experimental Amoebic Keratitis

Alongside of intranasal inoculation, anesthetizing of other three groups of rats was performed by Rompun and ketamine HCl intramuscularly, based on Polat et al study (14). After that, right eye of each rat as control and left eye as test were scraped by a sharp thickness blade, about 2-3 mm and then 1ml of solution containing 1×10^6 cyst was inoculated into stroma of each rat by an insulin syringe. Distillated water was inoculated into right eye as a control sample and all rats were evaluated up to 60 days post inoculation. Eyes of rats were transferred to the laboratory for cornea section and then stained by H&E. All of corneas were cultured in Bactoagar medium with *Escherichia coli*. Process of incision on rat eyes was a quotation of Polat and colleagues 2007 method (14). This work has been approved in research Ethics Committee of Tehran University of Medical Sciences, Iran.

Results

The presence of *Acanthamoeba* spp. was confirmed by morphological and molecular based approaches. Double walled cysts with star shape endocyst and flat trophozoites were observed in the plates. The presence of spine like structures called Acanthapodia were the main characteristic of *Acanthamoeba* spp. trophozoite. Axenic cultures were performed successfully after several months (Fig. 1). Axenic cultures were continuously performed for 12 months. All isolates (HS, KP and WD) showed a 500 bp PCR product band, which is specific for *Acanthamoeba* genus. Homology analysis confirmed that all isolate were belonged to T4 genotype with high homology. The accession numbers were shown in Table 1.

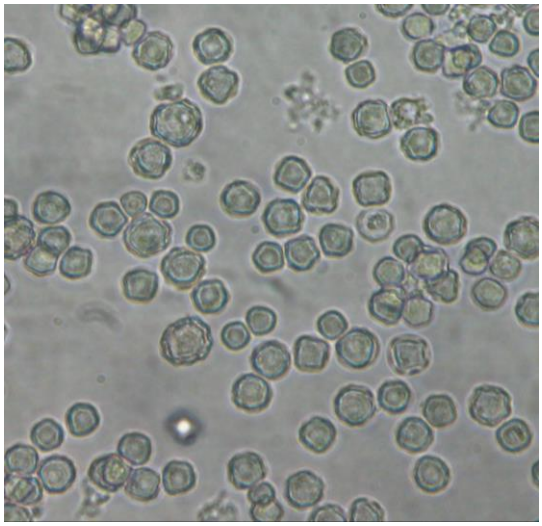

Fig. 1: Clinical *Acanthamoeba* isolate cysts in axenic medium (PYG), magnification X200

In vitro test

In vitro tests including osmotolerance assay revealed that none of isolates could grow up in concentration more than 0.5M. In thermotolerance test all of isolates grew in 30 °C, however, in 37 °C only HS isolate and in 40 °C none of isolates grew (please see table 1). Indeed, the in vitro test revealed that the isolates were not having a high potential pathogenic ability.

In vivo tests

Eyes of all rats examined macroscopically every 2 days. As expected there were no sign of infection in the right eyes of rats in the control groups. However, the signs of inflammatory reactions due to incision were observed in the first week. Nevertheless, inflammations were healed within 2 weeks. Slides of cornea and brain section were stained by H&E and examined by light microscope precisely. None of isolates was infected to cyst of *Acanthamoeba*. The cultures of brain and eye samples were surveyed by light microscope (magnification X100) as well every 2 days for 3 weeks but they did not represent any sign of *Acanthamoeba* growth. The results of in vivo tests confirmed in vitro results as well.

Table 1: In vitro and in vivo pathogenicity surveys of the T4 isolates from different sources

Code number	Source	GenBank Accession No.	Temperature tolerance assay			Osmotolerance assay			In vivo test
			30°C	37°C	40°C	0.5M	1M	1.5M	
1	KP	JQ903617	+	_	_	+	_	_	_
2	WD	HQ833407	+	_	_	+	_	_	_
3	HS	JN585814	+	+	_	+	_	_	_

KP = Keratitis Patient; WD = Windows Dust; HS = Hot Spring water

Discussion

In the present study, pathogenicity of *Acanthamoeba T4* genotype was evaluated by in vitro and in vivo tests. This is the first study regarding pathogenic potential of T4 genotypes in Iran. In vitro tests were done using mannitol and thermal tests, which showed that none of three isolate were a highly pathogenic strains. In vivo tests were also confirmed the in vitro assays in which none of the isolates could develop *Acanthamoeba* related infections in wistar rats. So far 17 genotypes of *Acanthamoeba* have been identified. Several genotypes including T3, T2, T4 and T6 were isolated from clinical cases such as Amoebic Keratitis and Granulomatose Encephalitis and Cutaneous lesion. On the other hand, some genotypes have not isolated from clinical samples such as T10 (20). It is important to mention that T4 genotype is the most common causative agent of keratitis and non-keratitis *Acanthamoeba* infections in Iran and worldwide (21-24). Various researches showed that T4 genotype is the predominant type in their samples (16, 25, 26). Moreover, Booton et al.. showed that isolates obtained from brain, cere-

brospinal fluid (CSF), nasal, skin and lung were belonged to T4 genotype (21). However, the main reason of abundant cases due to T4 genotype is unclear yet. Some researchers mentioned that the worldwide distribution and high transmissibility nature of the mentioned genotype might be one explanation of their predominance nature. Environmental strains have been isolated from wide variety of niches such as soil, water, dust, contact lenses and their cleaning solution and animal feces (2-4, 16).

Several studies showed correlation between growth in high temperature and osmolarity with pathogenicity of *Acanthamoeba* (16, 27, 28). However, temperature tolerance character probably plays less importance role in AK. Since average temperature of human eye is 34 °C (16, 21). On the other hand, temperature tolerance isolates has most important role in GAE, because body temperature of human is 37 °C (29). In the present study, all of three isolates grew in 30 °C and 0.5 M mannitol, but only HD isolate grew in 37 °C. All of isolates failed to grew in 40 °C, 1 M and 1.5 M mannitol. One hypothesis regarding the growth of HS isolate in 37 °C, could be due to its survival in high temperature of hot spring and amoeba adoptation to growth in higher temperature. It is interesting to mention that keratitis and GAE did not develop in wistar rats. These results are in consistent with Khan et al. who indicated that some T4 isolates have less pathogenicity effects on in vivo and in vitro tests (27). On the other hand, most of *Acanthamoeba* T4 isolates demonstrated cytopathic effects on cell culture medium (16). It is reported that cell culture can intensify pathogenicity of *Acanthamoeba*. However, long-term axenic culture can decrease the pathogenicity (30). In the present study, all isolates were cultured axenically for a long time (unpublished data) and we decided to evaluate their potential pathogenicities. This is important to mention that our isolate were axenied between 10-12 month after initial culture and after achieving purified culture we have performed physical based assays and in vivo tests. Although walochnic reported axenic cultur for long term did not have effect on pathogenicity of *Acanthamoeba* isolates (16). Our results are in concordance with finding of Stevens et al., which demonstrated that axenic culture for long term could attenuate the pathogenicity test of *Acanthamoeba* (30). None of our isolates developed to keratitis and GAE in wistar rats. This is due to low pathogenicity of *Acanthamoeba* isolates.

Conclusion

Amoebic keratitis and CNS disorders related to *Acanthamoeba* are multi-factorial cascades. Our results indicated that long-term axenic cultivation and *Acanthamoeba* sources could affect the pathogenicity and distinctive behavior of *Acanthamoeba* T4.

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