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ORIGINAL PAPER

Experimental infection of T4 *Acanthamoeba* genotype determines the pathogenic potential

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Abstract T4 is the *Acanthamoeba* genotype most related to cases of granulomatous amoebic encephalitis (GAE) in immunocompromised patients and of keratitis in contact lens wearers. The determination of the pathogenic potential of *Acanthamoeba* clinical and environmental isolates using experimental models is extremely important to elucidate the capacity of free-living organisms to establish and cause disease in hosts. The aim of this study was to compare and evaluate the histopathology and culture between two different routes of experimental infection of T4 *Acanthamoeba* isolated from environmental and clinical source in mice (intracranial and intraperitoneal). Swiss isogenic healthy mice were inoculated with 10^4 trophozoites by intracranial (IC) and intraperitoneal (IP) routes and observed during 21 days. The brains from animals inoculated by the IC route were collected and from the animals of the IP inoculation group, the brains, livers, kidneys, spleens, and lungs were removed. The organs were prepared and appropriately divided to be evaluated with histopathology and culture. There was no significant difference between the inoculation routes in terms of isolates recovery ($\chi^2 = 0.09$; $p = 0.76$). In the IC group, isolate recovery rate was significantly higher in histopathology than the one achieved by culture ($\chi^2 = 6.45$; $p < 0.01$). Experimental infection revealed that all isolates inoculated could be considered invasive because it was possible to recover evolutive forms of *Acanthamoeba* in both routes. This work represents the first in vivo pathogenicity assay of primary isolation source in Central region of Brazil showing in vivo pathogenicity and hematogenous spread capacity of these protozoa, improving the knowledge on free-living amoebae isolates.

Keywords *Acanthamoeba* · Experimental infection · T4 · Intracranial · Intraperitoneal

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Introduction

Acanthamoeba is a ubiquitous free-living amoebae genus that is able to brake down natural barriers and cause disease in humans and animals. These amoebae can develop its active form in many environments, but when infecting an immunocompromised host may cause granulomatous amoebic encephalitis (GAE), as well as keratitis in contact lens wearers (Khan 2006). Ertabaklar et al. (2007) described a case of *Acanthamoeba* keratitis in a immunocompetent individual with no history of contact lens wear caused by a T4 genotype *Acanthamoeba*. Koltas et al. (2015) has demonstrated that tap water is an important source of *Acanthamoeba* contamination in cases of *Acanthamoeba* keratitis in non-contact lens wearers.

The GAE is a progressive infection of the central nervous system (CNS) and usually occurs in immunocompromised patients after nasal mucosa penetration followed by hematogenous spread (Mortazavi et al. 2010; Siddiqui and Khan 2012). The determination of the pathogenic potential of *Acanthamoeba* clinical and environmental isolates using

experimental models is extremely important to elucidate the capacity of free-living organisms to establish and cause disease in hosts. Studies addressing the pathogenic potential and the use of molecular biology technologies for the identification of environmental samples worldwide have been very effective (De Jonckheere 1980; Gianinazzi et al. 2009; Alves et al. 2012; Mirjalali et al. 2013).

There are several direct and indirect pathogenicity factors of the *Acanthamoeba* genus. Among the direct factors are contact-dependent mechanisms such as adherence to host cells and phagocytosis of these cells by trophozoites. The contact-independent factor is the production of extracellular proteases. Pathogenicity indirect factors also include morphology, growth in high osmolarity, and at high temperatures, growth in different pH ranges, drug resistance, ubiquity, and host-linked factors (Khan et al. 2001; Khan and Tareen 2003; Siddiqui and Khan 2012; Alves et al. 2015; Baig 2015). Tawfeek et al. (2016) reported the isolation of pathogenic T4 genotype *Acanthamoeba*, among other genotypes, from sources such as keratitis, water, soil, and dust in Cairo, Egypt. The pathogenicity was determined through the osmotolerance, thermotolerance, and zymography analysis.

Experimental infection is widely used to demonstrate the pathogenicity of *Acanthamoeba* environmental isolates and the most experimental models used are mice, hamsters, pigs, rabbits, and locusts (Clarke and Niederkorn 2006; Mortazavi et al. 2010; Gianinazzi et al. 2010; Feng et al. 2015). *Acanthamoeba* has the ability to penetrate mucosa and to determine repeated damage in many organs, breaking down natural barriers such as blood brain barrier, which makes the nasal cavity one of the most infected sites (Siddiqui and Khan 2012). Indeed, these amoebae can multiply in the nasal cavity or in the peritoneal cavity (if experimentally inoculated by these routes), increasing the virulence of a small inoculum before CNS invasion (Alves et al. 2015). The virulence of 36 isolates has been demonstrated by the association of cytopathic effect and experimental infection by intracerebral (IC) inoculation followed by intranasal (IN) inoculation (De Jonckheere 1980). Many characteristics of the *Acanthamoeba* infection in mice have been described in the literature, such as the incubation period, clinical presentation, CNS dissemination, affected organs, host response, and developmental forms recovered from brain tissue (Martinez and Visvesvara 1997; Siddiqui et al. 2011; Massilamany et al. 2014). However, the experimental infection of clinical and environmental isolates with different routes of inoculation not only was able to reveal the ability of these protozoa to establish on target tissues but also demonstrated the hematogenous spread.

Therefore, the aim of this study was to compare and evaluate the histopathology and culture between two different routes of experimental inoculation of *Acanthamoeba* spp. in mice—intracranial and intraperitoneal.

Material and methods

This experiment was approved by the Ethics Committee of Animal Use (CEUA), located at the Institute of Biological Sciences (University of Brasília—UnB), protocol number UnBDOC 43036/2010.

Acanthamoeba isolates

Two T4 genotype isolates (UnB 11 and IP1S1) from our previous work (Alves et al. 2012); a keratitis T4 sample isolate (CorSP—genBank ID1517397) and the *Acanthamoeba polyphaga* (ATCC 30461) were used. Isolation and culture maintenance of *Acanthamoeba* isolates were performed as previously described by Alves et al. (2012). Trophozoites with 72 h of growth in the YAS liquid medium quantified by hemocytometer were used for the inoculation in mice.

Animals

Swiss isogenic healthy mice (male and female, $n=48$), with 15 days of life, taken from the UnB Medical School animal facilities, weight ranging from 10 to 15 g, were used for this study. The animals were taken into a laminar flow chamber and anesthetized by intraperitoneal injection of ketamine (80 mg/kg) + xylazine (10 mg/kg). After anesthesia, 30 uL of each culture containing 10^4 trophozoites were directly inoculated into the intracranial space (IC), guided by palpation (in the sagittal suture) and into the peritoneum cavity (IP) in six animals. Three animals were inoculated with a sterile saline solution in order to constitute a negative control group.

The mice were daily observed during 21 days to detect physiological changes such as ataxia and weight loss, as well as possible death. If any of these symptoms were present, the animals were sacrificed; tissue samples were collected and submitted to histopathology and culture attempting trophozoites recovery.

After this period, the remaining mice were anesthetized and sacrificed by injection of 1 mmol/kg of potassium chloride. To observe the presence of amoeba in the central nervous system, from animals inoculated by the intracerebral route only the brains were collected and from the animals of the IP inoculation group the following organs were removed: brain, liver, kidneys, spleen, and lung. The organs were divided for histopathology and culture.

Histopathology

The 10 % formalin fixed tissues were embedded in paraffin, sectioned at 5 μm in thickness and stained with hematoxylin-eosin. Trophozoites present in tissue were photographically documented (Sony Cyber-shot® camera, Sony Corp., China)

in the Laboratory of General Pathology, Federal University of Goiás (UFG).

Culture

The organs were macerated, seeded onto non-nutrient agar plates (1.5 %) covered with heat-inactivated *E. coli* and observed to evaluate the presence of developmental forms of *Acanthamoeba*. The plates were sealed with Parafilm (Brand GMBH + CO KG, Wertheim, Germany) and daily monitored for the detection of cysts and/or trophozoites, during 14 days (Ramirez et al. 2006; Rivera et al. 1993; Cerva 1971).

Evaluation of pathogenicity

In each route of inoculation, the isolates were classified according to their pathogenicity and invasiveness, as follows: (a) pathogenic—those directly related to physiological changes and/or death of at least one of the six mice and that also presented positive histopathology and/or positive culture; (b) invasive—those not directly related to physiological changes and/or death of animals during the study period (21 days), but associated with positive histopathology and/or positive culture; (c) non-pathogenic—those not related to physiological changes and/or death nor to positive histopathology and/or positive culture (Ramirez et al. 2006).

Statistical analysis

The differences between the routes of inoculation and recovery of the isolates were analyzed using the chi-square test (χ^2), with the Statistica 6.0 software (StatSoft Inc., Tulsa, OK, USA).

Results

Experimental infection (IC and IP) revealed that all isolates inoculated could be considered invasive because they were observed at histopathological examination of the brain tissues taken from the IP group animals (Table 1). There was no significant difference between the inoculation routes in terms of recovery of the isolates ($\chi^2 = 0.09$; $p = 0.76$).

Experimental infection by intracranial inoculation of *Acanthamoeba* spp. trophozoites

In the IC group isolates recovery rate was significantly higher in histopathology than the one achieved by culture ($\chi^2 = 6.45$; $p < 0.01$). All isolates showed positive histopathology in at least one animal (Table 2, Fig. 1). The UnB11 and IP1S1 isolates showed positive histopathology in two (30 %) of the six animals and the standard strain ATCC 30461 was positive in one animal (17 %). The CorSP isolate was the only one associated with death (all inoculated animals died within 24 h after inoculation), its developmental forms were observed on histopathology, besides being the only one recovered by culture (Table 2, Fig. 1c, d).

Experimental infection by intraperitoneal inoculation of *Acanthamoeba* spp. trophozoites

There was no statistical difference between the recovery of isolates by histopathology or culture ($\chi^2 = 0.10$; $p = 0.75$). All isolates showed positive histopathology in the brain tissue taken from at least one inoculated animal, demonstrating the presence of amoeba inside the CNS, attesting its invasiveness. The UnB11 isolate and the standard strain ATCC 30461 showed positive histopathology and culture in the kidney tissue taken from one of the inoculated animals. Three of the six animals inoculated with the pathogenic CorSP isolate died within 48 h. One of these animals showed spleen and liver positive histopathology and culture, as well as positive histopathology of lung, kidney, and brain tissues (Figs. 2 and 3a, b). The standard strain ATCC 30461 not only was observed in brain and spleen tissues histopathology but also was the only isolate that presented positive brain tissue culture (Table 3, Fig. 3c).

Table 1 Classification of *Acanthamoeba* isolates according to pathogenicity and invasiveness

Isolate	Intracerebral				Intraperitoneal				Classification
			Recovery				Recovery		
	AI[a]	M[b]	H[c]	C[d]	AI[a]	M[b]	H[c]	C[d]	
UnB11	6	N[f]	+	−	6	N[f]	+	+	Invasive
IP1S1	6	N[f]	+	−	6	N[f]	+	−	Invasive
CorSP	6	S[g]	+	+	6	S[g]	+	+	Pathogenic and invasive
ATCC 30461	6	N[f]	+	−	6	N[f]	+	+	Invasive

AI[a] inoculated animals, *M*[b] death, *H*[c] histopatology, *C*[d] culture, *N*[f] absence of death, *S*[g] death was observed, + cysts and/or trophozoites detected, − cysts and/or trophozoites not detected

Table 2 Recovery percentage of evolutive forms of *Acanthamoeba* isolates inoculated by intracranial route by histopathology and/or culture.

Isolates	H^c ($n^e = 6$)	C^d ($n^e = 6$)
UnB11	2 (33 %)	0
IP1S1	2 (33 %)	0
CorSP	6 (100 %)	3 (50 %)
ATCC 30461	1 (16 %)	0

H^c histopathology of brain tissue, *C^d* culture, *n^e* inoculated animals

Discussion

This study determined the invasiveness and pathogenicity of *Acanthamoeba* isolates through two different experimental inoculation routes in mice. Amoebae of the *Acanthamoeba* genus are soluble in bile and, therefore, cannot cause infection if they are ingested (Culbertson et al. 1959). Our study demonstrated the hematogenous spread after intraperitoneal inoculation.

All isolates used in experimental infection in this study were considered invasive and belong to T4 genotype (Alves et al. 2012) which has been mostly associated to encephalitis and keratitis (Siddiqui and Khan 2012). Other study has also isolated the T4 genotype from soil samples (Reyes-Batlle et al. 2016) showing that this genotype is widely spread in the environment. In a previous survey using in vitro temperature tolerance and osmotolerant tests to evaluate pathogenicity, UnB 11, one of the isolates used in this study, was considered thermotolerant. Other two isolates, IP1S1 and ATCC 30461, were considered osmotolerant and thermotolerant (Alves et al. 2012). According to the literature, in vivo assays generally corroborate the findings obtained in vitro

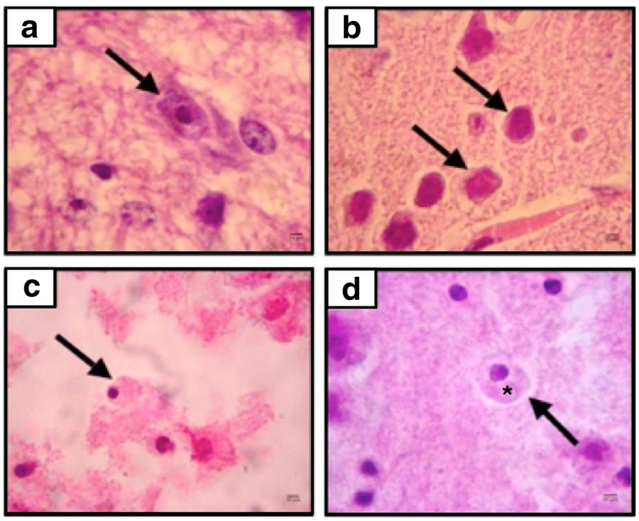

Fig. 1 Histological sections of the brains of animals of IC group. **a** Trophozoite (*arrow*) of UnB11 soil isolate. **b** cysts (*arrows*) of IP1S1 pool isolate. **c** and **d** trophozoites (*arrow*) isolated from keratitis CorSP with evident vacuole (*). Optical microscope; increase: ×1000; scale = 10 μm

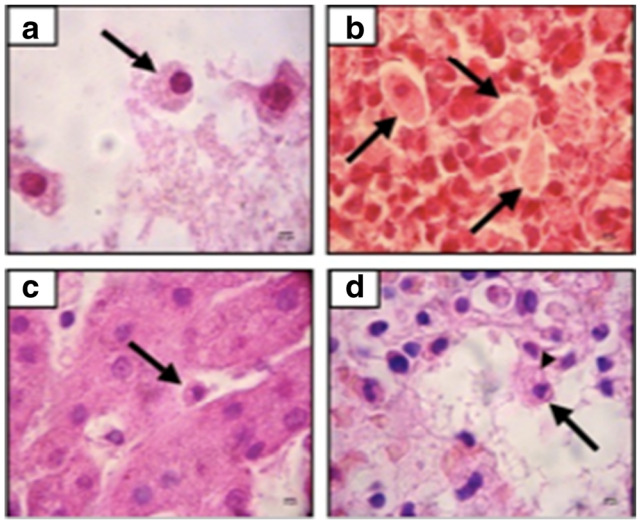

Fig. 2 Trophozoites of CorSP isolate (*arrows*) with vacuole (*arrowhead*) observed in histological sections of the organs taken from the animals of IP group. **a** Brain. **b** Spleen. **c** Liver. **d** Lung. Optical microscope; increase: ×1000; scale = 10 μm

(Gianinazzi et al. 2009; De Jonckheere 1980). In Belgium, growth characteristics, cytotoxic effect in cell cultures and virulence of 19 species of *Acanthamoeba* genus from various environmental isolates were evaluated in mice (De Jonckheere 1980). In a pathogenicity assay in Mexico, 6.5 % of the intranasally inoculated isolates were classified as pathogenic, 29 % as invasive and 64.5 % as non-pathogenic (Ramirez et al. 2006). In Switzerland, *Acanthamoeba* isolates from a heated indoor swimming pool were incubated at 37 °C, being considered as thermotolerant those that presented growth, which has been confirmed by in vivo pathogenicity tests using the intranasally inoculation in mice (Gianinazzi et al. 2009). Geisen et al. (2014) studied 65 *Acanthamoeba* isolates from soil from different locations in the Netherlands, Sardinia and Tibet, and the only pathogenic isolate was classified in the T4 genotype presenting thermotolerance and cytopathogenic effect on culture cells.

In this study, we used histopathology to detect the infection in different sites and culture medium to recover the isolates after the experimental inoculation. The total observation period was 21 days. The use of various techniques for the recovery and longer observation periods may enhance the detection of pathogenic isolates (De Jonckheere 1980; Ramirez et al. 2006). In a study using PCR to detect the presence of *Acanthamoeba* in tissues samples taken from infected animals by intranasal route, PCR products were found in the lungs and brains tissues of all infected immunosuppressed animals (Gianinazzi et al. 2009).

Although death was observed only in the animals inoculated with the CorSP isolate, the detection of cysts and trophozoites of other isolates shows amoebae abilities to cross the blood brain barrier and to establish inside the target organ, according to the results found in the IP group.

Table 3 Number of animals inoculated by intraperitoneal route with evolutive forms of *Acanthamoeba* isolates recovered on to the organs by histopathology and/or culture

Isolates	Organs ($n^e = 6$)									
	B^h		L^j		Li^k		S^l		K^m	
	H^c	C^d	H^c	C^d	H^c	C^d	H^c	C^d	H^c	C^d
UnB11	3 (50 %)	–	–	–	–	–	1 (16 %)	1 (16 %)	–	2 (33 %)
IP1S1	1	–	–	–	–	–	–	–	–	–
CorSP	1		1	1	1	2	1 (16 %)	2	–	1
ATCC 30461	2	1	–	–	–	–	1 (16 %)	–	–	2 (33 %)

B^h brain, L^j lung, Li^k liver, S^l spleen, K^m kidney, H^c histopathology, C^d culture, – negative histopathology and culture in the organs, n^e inoculated animals per isolate

Acanthamoeba cysts are able to survive in the peritoneal cavity showing its potential for hematogenous dissemination (Alves et al. 2015). In the 1980s, it was demonstrated that the cytopathic effect was not followed by pathogenicity in mice intranasally inoculated with isolates of *Acanthamoeba*, showing that experimental models may differ in sensitivity and that the route of inoculation may also influence pathogenicity (De Jonckheere 1980).

In animals from the IC group, the presence of amoebae in the brain tissue indicates its ability to remain alive and to develop its active evolutive form (trophozoite) in the organ where they were inoculated. In Korea, *Acanthamoeba* isolates maintained in culture from the primary isolation demonstrated weaker virulence when compared to those isolated from brain tissue of mice belonging to the intranasally inoculated group. Moreover, isolates that had been present at least for two times in the brain tissue of infected animals induced early death and demonstrated higher mortality rate than isolates from primary isolation (environmental sources) maintained in culture (65.5 vs. 2.8 %, respectively) (Im et al. 1999).

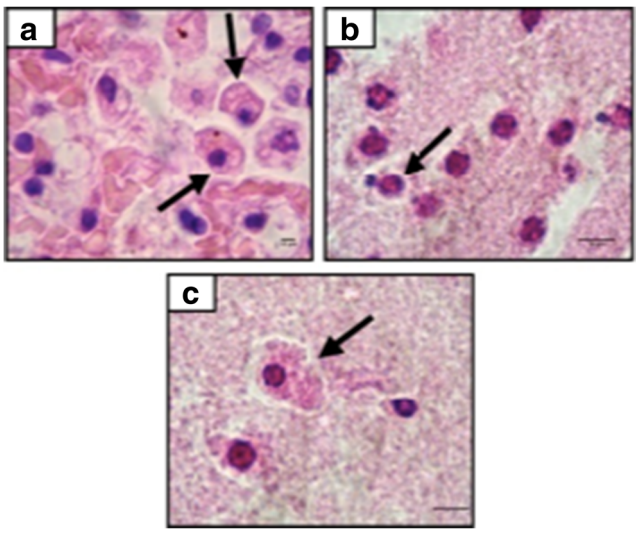

Fig. 3 Trophozoites observed in histological sections of the organs taken from animals of the IP group. **a** Trophozoites of CorSP isolate in the lung. **b** Cyst of CorSP isolate with wrinkled wall (*arrow*) in the brain. **c** Trophozoite of the standard strain ATCC 30461 inside the brain. Optical microscope; increase: ×1000; scale = 10 μm

This study revealed the presence of three isolates (two environmental and one from a patient with keratitis) that have exhibited their pathogenic traits by being osmotolerant and thermotolerant. Some of them also showed invasive features once they invaded the bloodstream and were observed in histopathological examinations (IC and IP groups). Besides that, in regards to the CorSP isolate, even death was observed. Despite the limitations inherent in working with experimental models, it was possible to demonstrate the ability to spread hematogenously (in regards to the CorSP isolate), which resulted in death of animals, with subsequent recovery of its developmental forms in various organs and tissues of mice.

Given the data presented in this study, it is possible to infer that there are potentially pathogenic strains in the studied environments of Brasilia, DF, and therefore, there is a risk that people attending swimming pools could get into contact with these pathogens (Khan 2006). Furthermore, if the relationship between direct and indirect virulence factors and the host immune system favors the development of the pathogenic potential of these amoebae, it is likely to observe the emergence of cases of human infections caused by *Acanthamoeba*, especially in higher susceptibility conditions, as occurs in diabetic individuals, human immunodeficiency virus infection, malnourished individuals, immunosuppressed patients, alcohol addicts, among others. It is noteworthy that the reasons and the mechanisms involved in leaving the free-living state to assume the parasitic and pathogenic behavior still remain unknown (Gianinazzi et al. 2009).

In recent decades, the *Acanthamoeba* genus is attracting growing interest in the scientific community (Khan 2006; Gianinazzi et al. 2009; Siddiqui et al. 2011), mainly by the multiple roles they play in the ecosystem, acting at the same time as predator and vector/reservoir (the Trojan horse mechanism) of microorganisms (Siddiqui et al. 2011).

Therefore, the invasive capacity of UNB 11 (soil) and CorSP (keratitis) isolates was demonstrated by the presence of developmental forms in histopathological examinations and their recovery in culture dishes was successfully

accomplished. This work represents the first in vivo pathogenicity assay of primary isolation source in Central region of Brazil showing in vivo pathogenicity and hematogenous spread capacity of these protozoa and improves the knowledge of free-living amoeba pathogenic potential.

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Compliance with ethical standards This experiment was approved by the Ethics Committee of Animal Use (CEUA), located at the Institute of Biological Sciences (University of Brasília—UnB), protocol number UnBDOC 43036/2010.

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