UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

CARACTERIZAÇÃO GENOTÍPICA DE ISOLADOS AMBIENTAIS DE *Acanthamoeba* spp. E ESTUDOS PROTEÔMICOS DE FORMAS TROFOZOÍTICAS VIRULENTAS E AVIRULENTAS DE *Acanthamoeba polyphaga*

TESE DE DOUTORADO

KARIN SILVA CAUMO

PORTO ALEGRE, RS, BRASIL

MAIO, 2013

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Tese submetida ao Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do grau de Doutor.

Orientadora: Dra. Marilise Brittes Rott

Co-orientador: Dr. Henrique Bunselmeyer Ferreira

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CARACTERIZAÇÃO GENOTÍPICA DE ISOLADOS AMBIENTAIS DE *Acanthamoeba* spp. E ESTUDOS PROTEÔMICOS DE FORMAS TROFOZOÍTICAS VIRULENTAS E AVIRULENTAS DE *Acanthamoeba polyphaga*[1]

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Orientador: Dra. Marilise Brittes Rott

Co-orientador: Dr. Henrique Bunselmeyer Ferreira

RESUMO

Acanthamoeba spp. são patógenos protistas de vida livre, capazes de causar ceratite grave e encefalite granulomatosa fatal. Trofozoíto é a forma infectiva de *Acanthamoeba* spp. e pode provocar infecções em uma variedade de hospedeiros mamíferos e seres humanos, como resultado da complexa interação patógeno-hospedeiro. O dano causado por trofozoítos em infecções da córnea ou do cérebro é o resultado de vários mecanismos patogênicos diferentes não elucidados a nível molecular até agora. Neste trabalho, descrevemos os resultados da caracterização genotípica de isolados ambientais de *Acanthamoeba* spp. e a identificão por análises proteômicas do repertório de proteínas expressas por trofozoítos de *Acanthamoeba polyphaga*. Nossos resultados permitiram a caracterização de 13 isolados ambientais de *Acanthamoeba* spp. obtidos da água de piscinas, que foram classificados em nível de genótipo com base na análise da sequência do gene da subunidade menor do rDNA. Nove dos 13 isolados foram identificados como pertencentes ao genótipo T5, três ao genótipo T4 e um ao genótipo T3. Vários genótipos têm sido relatados em todo o mundo como agentes causadores de ceratite amebiana, incluindo os genótipos T3, T4 e T5. O presente estudo indica que o genótipo T5 é um contaminante comum na água de piscinas. Este trabalho também estabeleceu uma ampla análise do repertório de proteínas expressas por trofozoítos de *A. polyphaga*, baseado no conjunto de estratégias por 2-DE MS/MS e LC-MS/MS. Foram identificadas 192 proteínas não redundantes. Um mapa proteômico de referência de *A. polyphaga* na faixa de pH 3-10 foi produzido e de 370 spots resolvidos, 136 proteínas foram identificadas. A classificação funcional das proteínas revelou diversas proteínas com relevância potencial para a sobrevivência do parasita e infecção de hospedeiros mamíferos, incluindo proteínas de superfície e proteínas relacionadas aos mecanismos de defesa. Este estudo descreveu a primeira análise proteômica abrangente do estágio de trofozoíto de *Acanthamoeba* spp. e fornece bases para estudos prospectivos, comparativos e funcionais de proteínas envolvidas nos mecanismos moleculares de sobrevivência, desenvolvimento e patogenicidade de *Acanthamoeba* spp.

[1]Tese de Doutorado em Microbiologia Agrícola e do Ambiente - Instituto de Ciências Básicas e da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (141 p.) Maio, 2013.

GENOTYPIC CHARACTERIZATION OF ENVIRONMENTAL ISOLATES OF *Acanthamoeba* spp. AND PROTEOMIC STUDIES OF VIRULENT AND NON-VIRULENT FORMS OF *Acanthamoeba polyphaga*[1]

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ABSTRACT

Acanthamoeba spp. are free-living protist pathogen, capable of causing a blinding keratitis and fatal granulomatous encephalitis. Trophozoite is the form infective of *Acanthamoeba* spp. and can provoke infections in a variety of mammalian hosts and humans, as result of complex interaction host-pathogen. The damage caused by trophozoites in human corneal or brain infections is the result of several different pathogenic mechanisms not elucidated at the molecular level so far. Here, we describe the results of the characterization genotypic of environment isolates of *Acanthamoeba* spp. and we performed proteomic analysis to identify the repertoire of proteins expressed by trophozoites of an environmental strain of *Acanthamoeba polyphaga*. Our results allowed the characterization of 13 *Acanthamoeba* isolates from swimming pools water that were classified at the genotype level based on the sequence analysis of the small-subunit rDNA gene. Nine of the 13 isolates were genotype T5, three were genotype T4, and one was T3. Several genotypes have been reported worldwide as causative agents of keratitis, including genotypes T3, T4, and T5. The present study indicates that genotype T5 is a common contaminant in swimming-pool water. This work also established a comprehensive analysis of the proteins expressed by *A. polyphaga* trophozoites based on complementary 2-DE MS/MS and gel-free LC-MS/MS approaches. Overall, 192 nonredundant proteins were identified. An *A. polyphaga* proteomic map in pH range 3-10 was produced, with protein identification for 136 out of 370 resolved spots. Functional classification of identified proteins revealed several proteins with potential relevance for parasite survival and infection of mammal hosts, including surface proteins and proteins related to defense mechanisms. This study describes the first comprehensive proteomic survey of the trophozoite infective stage of an *Acanthamoeba* species, and provides foundations to prospective, comparative and functional studies of *Acanthamoeba* proteins involved in molecular mechanisms of survival, development, and pathogenicity.

[1]Doctoral thesis in Agricultural Microbiology, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (141 p.) May, 2013.

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AVL	Amebas de vida livre
ACN	Acetonitrila
AK	*Acanthamoeba* keratitis
ApAI	*A. polyphaga* after infection
ApPI	*A. polyphaga* prior infection
ATCC	American Type Culture Collection
BLAST	Basic local aligment search tool
cm	Centímetro
DB	Database
DDA	Data-dependent acquisition
dNTP	Desoxirribonucleotídeo trifosfato
DTT	Ditiotreitol
EAG	Encefalite amebiana granulomatosa
ELISA	Ensaio de imunoadsorção ligado a enzima
ESI	Ionisação por electrospray
EST	Marca de sequência expressa
FLA	Free-linving amoebae
g	Grama
GAE	Granulomatous amoebic encephalitis

GO	Gene onthology
IPG	Gradient de PH imobilizado
ITS	Espaçador interno transcrito
kDa	Quilodalton
h	Hora
Hz	Hertz
IEF	Focalização isoelétrica
KOG	Eukaryotic Orthologous Group
LC/MS-MS	Cromatografia líquida acoplada a espectrometria de massas em tandem
LSU	Subunidade maior
M	Molar
MALDI	Ionização/dessorção a laser assistida por matriz
MBP	Mannose binding protein
mg	Miligrama
mg/kg	Miligrama/kilograma
min	Minuto
MIP	Manose induced protein
MIP-2	Macrophage inflamatory protein 2
ml	Mililitro
mM	Milimolar
MS	Espectrometria de massas
MS/MS	Espectrometria de massas em tandem
Mw	Massa molecular
ng	Nanograma

NK	Natural killer
nl	Nanolitro
PAGE	Eletroforese em gel de poliacrilamida
pb	Pares de base
PBS	Phosphate-buffered saline
PCR	Reação em cadeia da polimerase
pH	Potencial hidrogeniônico
PHMB	Polihexametileno biguanida
Pi	Ponto isoelétrico
PVDF	Fluoreto de Polivinilideno
PYG	Proteose peptone, yeast extract, glucose
Q	Quadrupole
S	Segundo
SDS	Sodium dodecyl sulfate
SNC	Sistema nervoso central
SOD	Superóxido dismutase
SSU	Small-subunit
TFA	Trifluoroacetic acid
TOF	Time of flight
U	Unidade
UV	Ultra-violeta
V	Volts
x g	Força centrífuga
°C	Grau Celsius
µg	Micrograma

µl	Microlitro
µM	Micromolar
2-DE	Eletroforese bidimensional

1. INTRODUÇÃO

O estudo de organismos parasitários emergentes tem sido objeto de pesquisas em todo o mundo, especialmente em relação àqueles que trazem maiores riscos à saúde humana. Desta forma, a importância do estudo de espécies de *Acanthamoeba* como agentes etiológicos de doenças é ressaltada pelo aumento do número de casos descritos, especialmente os casos de ceratites e encefalites. A ceratite amebiana destaca-se pelo aumento da incidência e morbidade ocular face à popularização do uso de lentes de contato. Quando diagnosticadas tardiamente, as ceratites são caracterizadas pela complexidade terapêutica, podendo evoluir para quadros clínicos de úlceras de córnea, transplante da córnea infectada ou, eventualmente, a perda definitiva da visão. Apesar do número crescente de casos clínicos associados a infecções por *Acanthamoeba* spp. e o aumento do número de estudos científicos, pouco se sabe sobre a biologia destes parasitos, fatores de virulência que determinam a patogenicidade, especialmente no que diz respeito aos mecanismos de interação patógeno-hospedeiro. A determinação correta da taxonomia dentro do gênero é um fator essencial para que se possa chegar a generalizações e conclusões acerca da ecologia, patogenicidade e epidemiologia, relacionadas a qualquer patógeno, uma vez que a falta de linhagens bem definidas causa grandes equívocos na comparação de diversos casos estudados.

As análises baseadas em sequências do 18S rDNA são as maispromissoras para o estabelecimento de filogenias e para a classificação de espécies no gênero *Acanthamoeba*.

Estudos proteômicos vêm sendo realizados para diversos protozoários. Essas análises têm revelado a diversidade de proteínas expressas por diferentes parasitos, auxiliando na elucidação de mecanismos moleculares utilizados por cada um deles na interação com espécies hospedeiras, na manutenção da infecção e na identificação de biomarcadores potenciais para o diagnóstico, alvos vacinais e o desenvolvimento de novas drogas. Para *Acanthamoeba* spp. nenhuma análise proteômica global foi realizada até agora. A identificação de proteínas das formas invasivas de isolados de *Acanthamoeba* spp. pode levar a um melhor entendimento de quais são as moléculas envolvidas na patogenicidade (fatores de virulência), que poderão ser utilizadas como marcadores na diferenciação de isolados patogênicos e não patogênicos em estudos epidemiológicos, bem como fornecer informações importantes para o entendimento das características biológicas do parasito, mecanismos de evasão da resposta imune, estabelecimento e desenvolvimento da infecção.

Neste contexto, o presente trabalho teve por objetivos a caracterização genotípica de isolados ambientais de *Acanthamoeba* spp. e a realização de estudos proteômicos para a identificação do repertório de proteínas expressas por trofozoítos de um isolado ambiental de *Acanthamoeba polyphaga*, visando à identificação de proteínas possivelmente envolvidas na patogenicidade e/ou virulência e também proteínas antigênicas.

Os objetivos específicos do estudo foram:

1. Determinar o genótipo de isolados ambientais de *Acanthamoeba* spp., com base em sequências do 18S rDNA;
2. Estabelecer um mapa proteômico de referência para trofozoítos de *A. polyphaga*, incluindo a identificação de padrões de processamento pós-traducional;
3. Realizar a análise proteômica comparativa do repertório de proteínas expressas por trofozoítos do isolado ambiental de *A. polyphaga* com virulência atenuada por longo tempo de cultivo *in vitro* e com restauração da virulência por re-isolamento de trofozoítos de órgãos infectados após infecção *in vivo*;
4. Identificar proteínas antigênicas em extratos do parasito com potencial para o diagnóstico de infecções por *Acanthamoeba* spp.

2. REVISÃO BIBLIOGRÁFICA

2.1 Amebas de vida livre patogênicas

Amebas de vida livre (AVL) são protozoários unicelulares amplamente dispersos no ambiente (Trabelsi *et al.*, 2012). Ao longo das últimas décadas estes organismos ganharam atenção crescente da comunidade científica, devido aos diversos papéis no ecossistema e, em particular por causarem infecções oportunistas e não oportunistas graves em seres humanos e diferentes espécies de animais (Siddiqui & Khan, 2012). Estas amebas são organismos não bem adaptados ao parasitismo, já que podem causar infecções rapidamente letais sem transmissão entre hospedeiros e são conhecidos como anfizóicos, pois possuem a capacidade de viver como organismos de vida livre e eventualmente habitar um hospedeiro causando dano (Lorenzo-Morales *et al.*, 2013).

Sabe-se atualmente que dentre as AVL capazes de causar infecções em seres humanos, encontram-se diversas espécies do gênero *Acanthamoeba*, o amebo-flagelado *Naegleria fowleri*, *Balamuthia mandrillaris* e mais recentemente *Sappinia diploidea* (Visvesvara *et al.*, 2007). A importância destes microrganismos como agentes etiológicos é ressaltada pelo aumento do número de casos descritos, especialmente os casos de ceratites e encefalites, que são quase sempre graves, de difícil diagnóstico e não possuem terapêutica efetiva bem definida (Saxena *et al.* 2009; Visvesvara, 2010).

Espécies do gênero *Acanthamoeba* e *Balamuthia madrillaris* causam infecções oportunistas em indivíduos imunocomprometidos ou debilitados, tais como encefalite amebiana granulomatosa, lesões cutâneas, nasofaringeana, infeções pulmonar e renal; *Acanthamoeba* spp. também causam ceratite amebiana, uma infecção ocular não-oportunista, que atinge principalmente usuários de lentes de contato (Visvesvara, Moura *et al.*, 2007; Siddiqui & Khan, 2012). Dentre as quarenta espécies descritas para o gênero *Naegleria*, apenas *Naegleria fowleri* causa meningoencefalite aguda hemorrágica e fulminante, levando à morte indivíduos saudáveis com história de contato com água contaminada (Marciano-Cabral & Cabral, 2003; Visvesvara *et al.*, 2007). *Sappinia diploidea*, uma AVL normalmente encontrada em solo contaminado com fezes de alces, bisões e gado, foi identificada como espécie causadora de encefalite (Gelman *et al.*, 2001).

Com relação à taxonomia das AVL, a classificação clássica dividia os protozoários em quatro grandes grupos: Sarcodina (amebas), Mastigóforos (flagelados), Sporozoa (a maioria dos protozoários parasitas) e Infusoria (ciliados) (Visvesvara, *et al.*, 2007). Atualmente a Sociedade Internacional de Protozoologistas propôs uma nova classificação taxonômica, baseada em provas morfológicas, bioquímicas e filogenia molecular. De acordo com este novo esquema, os eucariotos tem sido classificados em seis grupos, ou super-grupos, chamados de Amoebozoa, Opisthokonta, Rhizaria, Archaeplastida, Chromalveolata e Excavata (Figura 1). Os quatro gêneros de AVL patogênicas foram classificados sob dois supergrupos, como segue: os gêneros *Acanthamoeba* e *Balamuthia* estão classificados no supergrupo Amoebozoa, subdivisão Acanthamoebidae; *Naegleria fowleri* no supergrupo Excavata, Heterolobosia:

Vahlkampfiidae; e o gênero *Sappinia* no supergrupo Amoebozoa, subdivisão Thecamoebidae: Flabellinea (Adl *et al.*, 2005; Trabelsi *et al.*, 2012).

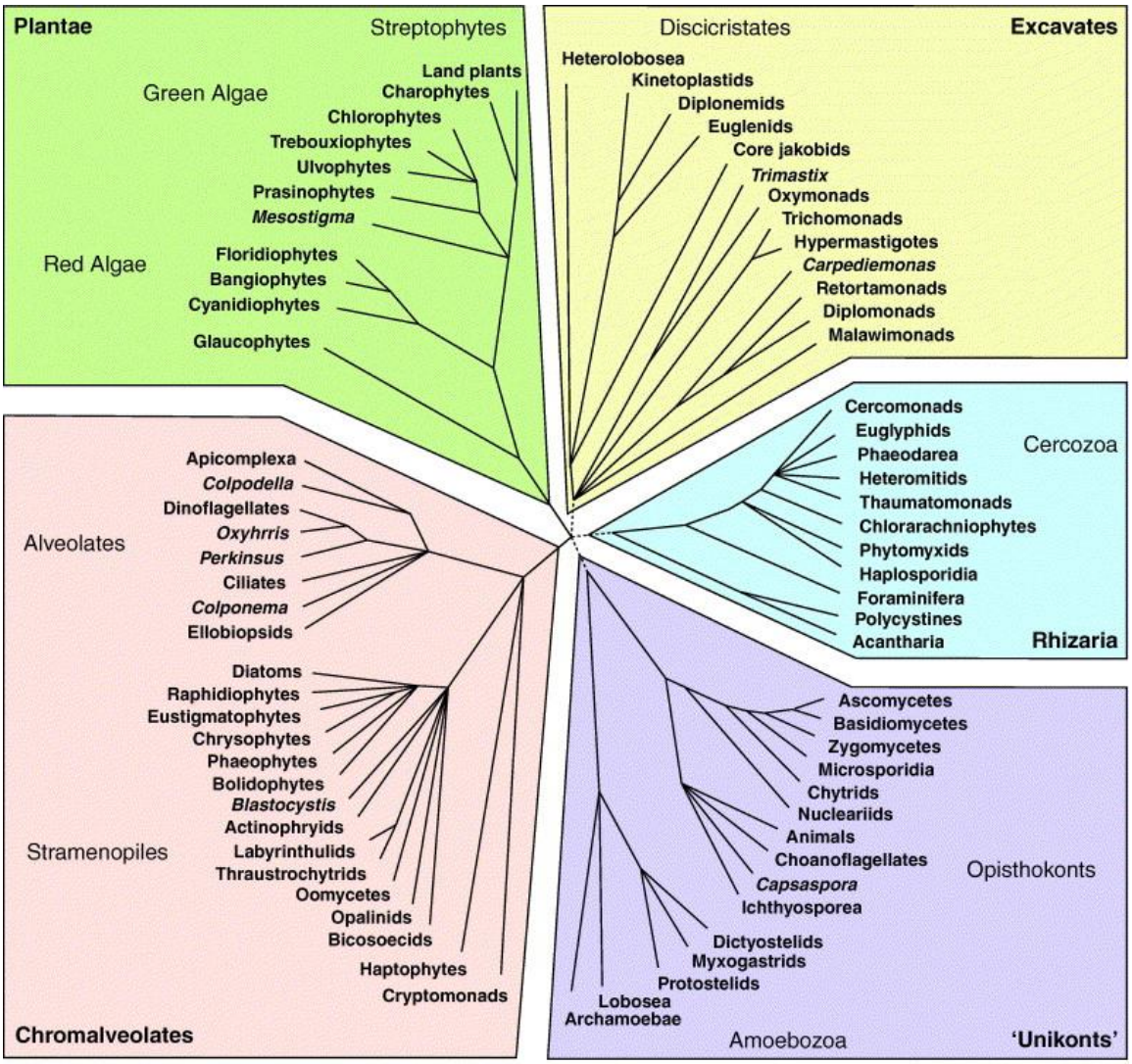

Figura 1. Árvores de eucariotos baseadas na nova classificação taxonômica proposta pela Sociedade Internacional de Protozoologistas. Fonte: (Keeling *et al.*, 2005).

2.2 Aspectos gerais de *Acanthamoeba* spp.

O primeiro isolado do gênero *Acanthamoeba* foi obtido de poeira em 1913 por Puschkarew e chamado de *Amoeba polyphagus* (Page, 1967). Em 1930, Castellani isolou uma ameba que estava contaminando uma placa de cultura de levedura, que foi

subseqüentemente chamada de *Acanthamoeba castellanii*. O interesse médico e epidemiológico com relação as AVL começou quando Culbertson *et al*. (1958), durante a produção de vacinas para a poliomielite, observaram o desenvolvimento de placas de algum organismo contaminante da cultura celular. O fluído dessas placas quando inoculado em ratos e macacos levava-os à morte por encefalite. Os contaminantes destas culturas eram cistos e trofozoítos de amebas, identificadas como pertencente ao gênero *Acanthamoeba*.

Dentre as AVL, há especial interesse no estudo de espécies do gênero *Acanthamoeba*, que são provavelmente os protozoários de vida livre mais abundantes na natureza, apresentam uma distribuição cosmopolita e têm sido isoladas de uma grande variedade de habitats (Schuster & Visvesvara, 2004; Caumo *et al.*, 2009; Carlesso *et al.*, 2010; Winck *et al.*, 2011; Siddiqui & Khan, 2012). Acantamebas apresentam papel potencial nos ecossistemas, devido a sua representatividade e atuação como portadores e reservatórios de bactérias, fungos e vírus (Greub *et al.*, 2005; Schmitz-Esser *et al.*, 2008). As acantamebas são bem reconhecidas como organismos que causam um amplo espectro de infecções tanto em seres humanos como em diferentes espécies de animais, cuja natureza depende do contato do potencial patógeno com determinados tecidos do hospedeiro e do *status* imunológico do mesmo (Schuster & Visvesvara, 2004; Khan, 2006). Acantamebas também são conhecidas como bons modelos para o estudo de aspectos básicos da biologia de células eucarióticas, devido ao seu tamanho relativamente grande, citoesqueleto bem desenvolvido, ativa motilidade e rápida multiplicação em cultura (Preston *et al.*, 2001; Siddiqui & Khan, 2012).

2.2.1 Morfologia e ciclo de vida de *Acanthamoeba* spp.

Em seu ciclo biológico, as *Acanthamoeba* spp. apresentam-se sob duas formas (Figura 2): a de trofozoíto, metabolicamente ativa, capaz de dividir-se, alimentar-se e invadir células do hospedeiro; e a de cisto, forma de resistência a condições ambientais adversas (Page, 1967). As infecções causadas por *Acanthamoeba* spp. acontecem quando trofozoítos ou cistos presentes no ambiente entram em contato com os olhos, com feridas na pele ou através do trato respiratório do hospedeiro, resultando no possível estabelecimento e proliferação das *Acanthamoeba* spp. no novo ambiente (Marciano-Cabral & Cabral, 2003).

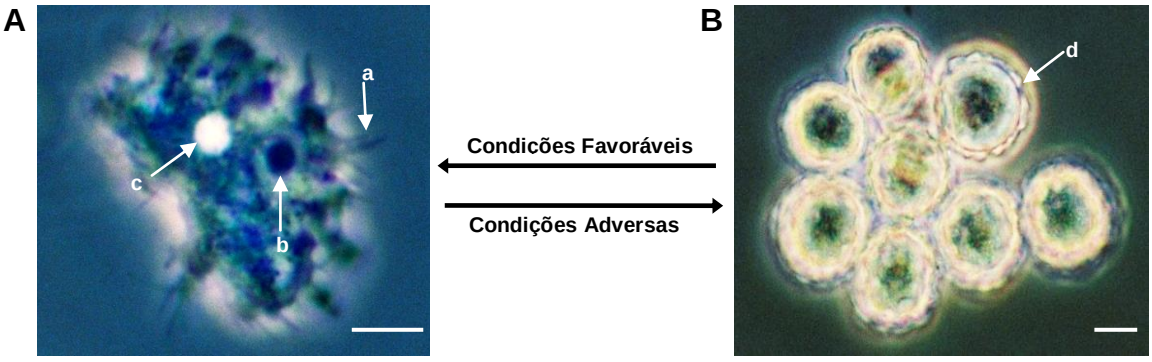

Figura 2. Ciclo de vida de *Acanthamoeba* spp. Nota-se no trofozoíto (A) a presença das expansões citoplasmáticas em forma de espinhos – acantopódios (a); Observa-se o nucléolo marcante (b) e a presença de vacúolo pulsátil (c). Cisto (B) com parede externa tipicamente enrugada, onde observa-se a presença da parede dupla (d, seta). Microscópio de contraste de fase. Aumento de 1000X. Barra = 5 µm
Fonte: Karin Caumo

Os trofozoítos se alimentam de bactérias, algas, fungos, leveduras, matéria orgânica ou por inclusão de partículas líquidas. A divisão celular é assexuada ocorrendo por fissão binária e não possuem um estágio flagelar (Schuster & Visvesvara, 2004). São células com tamanhos de 20 a 40 µm de diâmetro, variando significativamente entre

isolados pertencentes a diferentes espécies/genótipos (Lorenzo-Morales *et al.*, 2013). O citoplasma do trofozoíto de *Acanthamoeba* spp. é abundante e apresenta-se granuloso, com movimentos polidirecionais através da emissão de pseudópodes globosos e hialinos, de onde surgem finos pseudópodes semelhantes a espinhos, chamados de acantopódios. Os acantopódios, estruturas características do gênero, são projeções aciculiformes da membrana celular importantes para a alimentação e movimento celular. Geralmente os trofozoítos são uninucleados com um grande nucléolo central, entretanto, células multinucleadas são comuns quando *Acanthamoeba* spp. são mantidas em culturas líquidas (Marciano-Cabral & Cabral, 2003). O citoplasma apresenta diversos vacúolos, possui um vacúolo contrátil proeminente, que desaparece temporariamente e reaparece em um movimento de sístole-diástole, responsável pelo controle osmótico da célula, além de vacúolos digestivos. São organismos aeróbicos, mas seus trofozoítos podem se adaptar em ambientes com pouco oxigênio, durante um curto período de tempo (Visvesvara *et al.*, 2007; Edagawa *et al.*, 2009).

O encistamento das amebas é uma forma de proteção contra fatores adversos como a dessecação, falta de nutrientes e uma variedade de agentes químicos (desinfetantes e antimicrobianos) e físicos (calor, frio, radiação ultravioleta) (Aksozek *et al.*, 2002; Visvesvara *et al.*, 2007; Siddiqui & Khan, 2012). O tamanho dos cistos do gênero *Acanthamoeba* pode variar entre 5 e 20 μm de diâmetro. São compostos por celulose (não presente no estágio de trofozoíto), que representa 10% do peso seco total do cisto e diversas proteínas. Possuem duas paredes: o endocisto e o ectocisto. De forma geral, o ectocisto é mais esférico, enquanto o endocisto apresenta diversas formas, que dependendo do isolado, podem variar de triangulares a estreladas. Os cistos apresentam um opérculo, local por onde a ameba sai ao desencistar assim que as condições

ambientais se tornam favoráveis. Os dois envoltórios são separados entre si, mas se unem nos ostíolos, que são poros usados para monitorar as alterações ambientais (Lorenzo-Morales *et al.*, 2013).

2.2.2 Classificação de *Acanthamoeba* spp.

O gênero *Acanthamoeba* é um táxon que inclui pelo menos 24 espécies (identificadas com base em critérios morfológicos) distribuídas mundialmente, sendo que 12 delas (*Acanthamoeba astronyxis, A. castellanii, Acanthamoeba culbertsoni, Acanthamoeba divionesis, Acanthamoeba griffini, Acanthamoeba hatchetti, Acanthamoeba healyi, Acanthamoeba lenticulata, Acanthamoeba lugdunensis, A. polyphaga, Acanthamoeba quina* e *Acanthamoeba rhysodes*) são relacionadas a doenças em seres humanos (Booton *et al.*, 2005; Corsaro & Venditti, 2010; Visvesvara, 2010).

Durante muito tempo, a classificação de amebas do gênero *Acanthamoeba* esteve apoiada apenas em parâmetros morfológicos, que levavam principalmente em conta o tamanho e morfologia dos cistos. A classificação morfológica proposta por Pussard & Pons (1977) subdividiu o gênero em três grupos morfológicos distintos (I, II e III). As espécies definidas no grupo morfológico I (*A. astronyxis, Acanthamoeba comandoni* e *Acanthamoeba tubiashi*) são as que apresentam cistos de maiores dimensões (diâmetro médio >18 µm) e se caracterizam pela forma estrelada da parede cística interna (endocisto) e a parede externa (ectocisto) é mais ou menos esférica. No grupo II, encontram-se as espécies de *Acanthamoeba* mais amplamente e comumente isoladas (*A. castellanii, A. polyphaga, A rhysodes, A. griffini, A. hatchetti, Acanthamoeba triangularis, A. divionensis, Acanthamoeba mauritaniensis, A. lugdunensis* e *A. quina*), seus cistos possuem um tamanho médio com diâmetro de 18

μm ou menos, o endocisto pode apresentar-se de forma estrelada, oval, triangular ou quadrangular e o ectocisto segue mais ou menos o contorno do endocisto (Khan, 2006). O grupo III compreende as espécies cujos cistos medem aproximadamente 18 μm ou menos de diâmetro (*A. culbertsoni, A. healyi, Acanthamoeba jacobsi, A. lenticulata, Acanthamoeba palestinensis, Acanthameba pustulosa* e *Acanthamoeba royreba*), com endocisto arredondado ou globoso, nunca estrelado. O ectocisto é fino e liso ou fracamente franzido, justaposto ao endocisto (Khan, 2006; Visvesvara *et al.*, 2007). A identificação de espécies de *Acanthamoeba* empregando unicamente critérios morfológicos é muito subjetiva, uma vez que as características morfológicas dos cistos são influenciadas pelas condições ambientais e de cultivo. Alguns trabalhos relatam que mesmo controlando as condições de cultivo e de encistamento, observa-se que diferentes espécies apresentam características similares em seus cistos e que em cultivos monoclonais aparecem cistos de diversas morfologias (Visvesvara, 1991; Marciano-Cabral & Cabral, 2003).

Atualmente a identificação e taxonomia de *Acanthamoeba* spp. baseiam-se em filogenias moleculares que utilizam principalmente o sequenciamento da subunidade menor do DNA ribossômico. Vários fatores tornam 18S rDNA um alvo privilegiado para a análise molecular. Seu tamanho (cerca de 2.000 pares de bases) geralmente possibilita a obtenção de dados suficientes para a observação de sinal filogenético; grande quantidade de cópias no genoma, quase sempre evoluindo em conjunto, facilita o isolamento da molécula; ubiquidade, permitindo a construção de filogenias universais; presença de regiões mais e menos variáveis na mesma molécula, possibilitando a análise tanto de organismos proximamente relacionados (utilizando regiões de alta taxa de substituição), quanto pertencentes a reinos diferentes (utilizando regiões mais

conservadas), como também a grande quantidade de dados já gerados sobre essas sequências em diversos grupos de organismos, facilitando a análise comparativa.

No gênero *Acanthamoeba*, as unidades de repetição do cistron ribossômico (Figura 3) é de cerca de 12.000 pares de base (pb), contendo aproximadamente 600 cópias por genoma (Byers *et al.*, 1990). A subunidade maior (LSU rDNA) está subdividida em dois segmentos de 2.400 pb (LSU a) e 2000 pb (LSU b), separados por um espaçador interno transcrito (ITS), de cerca de 200 pb, que é removido do transcrito primário. O RNA da subunidade menor tem um tamanho de cerca de 2.300 a 2.700 pb dependendo da espécie.

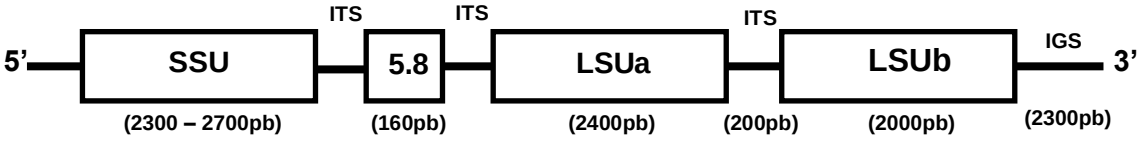

Figura 3. Esquema do cistron ribossômico em *Acanthamoeba* spp.

A classificação das espécies de *Acanthamoeba*, baseada na determinação de genótipos foi inicialmente estudada por Gast & Byers (1995), que demostraram a partir do desenvolvimento de sondas de oligonucleotídeos gênero-específicos, diferenças na identidade molecular entre isolados de uma mesma espécie de *Acanthamoeba* (Gast & Byers, 1995). A organização do gênero foi revolucionada quando Stothard *et al*. (1998), a partir da análise das seqüências do gene de diversos isolados de amebas, dividiram *Acanthamoeba* spp. em diferentes genótipos (numerados de T1 a T15). Observou-se, assim, que os grupos gênicos não tinham relação com a classificação por espécie baseados na classificação morfológica, evidenciando que a identificação molecular era mais precisa (Stothard *et al.*, 1998). Sequências da subunidade 16S do DNA

ribossômico mitocondrial também foram utilizadas com sucesso para análises filogenéticas, apresentando correlação com os dados observados na análise das sequências nucleares (Ledee *et al.*, 2003). Em conjunto, estes dados revelam que a separação por grupos gênicos reflete, de maneira apropriada, a história evolutiva do gênero.

Até o momento, 17 genótipos foram identificados, baseados no sequenciamento do 18S rDNA, nomeados T1 a T17 (Stothard *et al.*,1998; Schroeder *et al.*, 2001; Maghsood *et al.*, 2005; Corsaro & Venditti, 2010; Nuprasert *et al.*, 2010). Cada genótipo exibe 5% ou mais de divergência de sequências entre diferentes genótipos. Os genótipos T2, T3, T4, T5, T6, T11, T13, T14 e T16 têm sido isolados clinicamente como agentes causadores da ceratite amebiana, sendo o genótipo T4 associado à maioria das infecções humanas por *Acanthamoeba* spp., com aproximadamente 90% dos casos de ceratite ligados a este genótipo (Maghsood *et al*., 2005; Siddiqui & Khan, 2012; Maciver *et al.*, 2013). A tabela 1 apresenta a correlação entre genótipos e suas associações com patologias em humanos.

Tabela 1 - Genótipos de *Acanthamoeba* spp. e suas associações com a ocorrência de doenças humanas (ceratites e encefalites).

Genótipos de *Acanthamoeba* spp.	Doenças humanas associadas
T1	Encefalite
T2a	Ceratite e Encefalite
T2b	NA
T3	Ceratite
T4	Ceratite e Encefalite
T5	Ceratite e Encefalite
T6	Ceratite
T7	NA
T8	NA
T9	NA
T10	Ceratite e Encefalite
T11	Ceratite
T12	Encefalite
T13	NA
T14	NA
T15	Ceratite
T16	NA
T17	NA

NA: genótipo não associado a casos de doença em humanos.

Fonte: Adaptado de Siddiqui & Khan (2012).

2.2.3 Infecções causadas por *Acanthamoeba* spp.

Dentre as infecções mais importantes causadas por *Acanthamoeba* spp. destaca-se a ceratite amebiana, uma infecção não oportunista, associada principalmente a usuários de lentes de contato. De forma mais rara, *Acanthamoeba* spp. também poderão causar encefalite granulomatosa amebiana e infecções disseminadas em indivíduos imunocomprometidos.

2.2.3.1 Ceratite amebiana

A ceratite por *Acanthamoeba* spp. é uma infecção ocular grave, que se caracteriza pela perda gradual da visão, chegando, em alguns casos, a provocar cegueira. Ao contrário da encefalite, a ceratite amebiana ocorre em indivíduos imunocompetentes, principalmente em usuários de lentes de contato (Awwad *et al.*, 2007; Lorenzo-Morales *et al.*, 2013).

A ceratite amebiana é uma doença progressiva e dolorosa. Clinicamente os sintomas estão ligados à invasão e a degradação dos componentes da córnea, resultando em vermelhidão, lacrimejamento, fotofobia, opacidade e edema nas pálpebras (Marciano-Cabral & Cabral, 2003; Dart *et al.*, 2009; Lorenzo-Morales *et al.*, 2013). A dor relatada na ceratite amebiana, provocada por ceratoneurite radial, geralmente é muito intensa e não diretamente relacionada com o quadro clínico visual e ocorre pelo contato de amebas com as células nervosas no estroma da córnea (Clarke & Niederkorn, 2006a). Inicialmente as amebas são restritas ao epitélio da córnea, com erosões epiteliais puntiformes e tardiamente invadem o estroma, causando dano, opacidade e inflamação, provocados pela presença de neutrófilos, gerando um infiltrado em forma de anel, que é típico da ceratite amebiana (Figura 4) (Illingworth & Cook, 1998; Marciano-Cabral & Cabral, 2003; Clarke & Niederkorn, 2006b; Patel & Mcghee, 2009). Alterações na região posterior do olho, como retina e nervo óptico são raras (Illingworth & Cook, 1998). Além da formação do abcesso anelar, poderão ocorrer esclerite, glaucoma, catarata, perfuração da córnea e infecções microbianas secundárias (Lorenzo-Morales *et al.*, 2013).

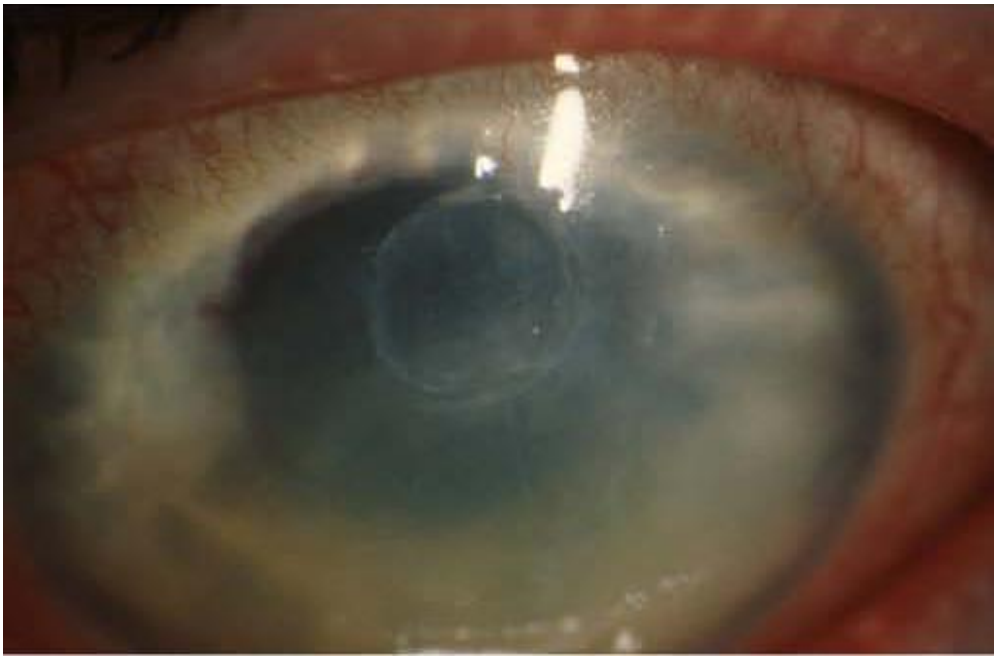

Figura 4. Ceratite por *Acanthamoeba* spp. em humano. Infiltrado neutrofílico em forma de anel e esclerite grave em caso avançado de ceratite amebiana.
Fonte: Dart *et al.* (2009)

O primeiro caso de ceratite amebiana em humanos foi descrito em 1973 na Inglaterra, seguido por publicação nos Estados Unidos (Nagington *et al.*, 1974; Jones et al., 1975). No Brasil, os primeiros casos foram descritos em 1988 (Nosé *et al.*, 1988). Os casos de ceratite amebiana diagnosticados aumentaram dramaticamente durante os últimos vinte anos, face à popularização do uso de lentes de contato (mais de 20 milhões de usuários apenas nos Estado Unidos) (Illingworth & Cook, 1998). Aproximadamente 83% dos casos de ceratite amebiana ocorrem em usuários de lentes de contato (Carvalho *et al.*, 2009), estimando-se de 1 a 60 casos de ceratite amebiana para cada 30.000 usuários de lentes de contato (Henriquez *et al.*, 2009). Nos Estados Unidos, Austrália, Itália, Nova Zelândia e Brasil, estudos têm demonstrado um significativo aumento do número de pacientes com ceratite amebiana (Thebpatiphat *et al.*, 2007; Sousa *et al.*, 2008; Ku *et al.*, 2009; Gatti *et al.*, 2010; Patel *et al.*, 2010). Nos

Estados Unidos calcula-se mais de 3.000 casos descritos (Qvarnstrom *et al.*, 2006). Na Inglaterra, a incidência é de 17 a 21 casos por milhão de usuários de lentes de contato (Awwad *et al.*, 2007). Existem poucos dados epidemiológicos descritos sobre a incidência de ceratite amebiana no Brasil, mas alguns relatos de casos são descritos, como a retrospectiva realizada por Carvalho *et al*. (2009) sobre a investigação em um centro de oftalmologia em São Paulo durante duas décadas, relatando um aumento do número de pacientes com ceratite amebiana ao longo dos anos. Aproximadamente 10 a 15% de casos de ceratite amebiana ocorrem em pessoas que não usaram lentes de contato (Illinworth & Cook, 1998) e está associada com trauma ou exposição à água ou solo contaminado, frequentemente em trabalhadores rurais, como tembém também após cirurgias oculares.

Alguns estudos associam a maior incidência de ceratite amebiana em alguns países aos seguintes fatores: utilização de lentes de contato (mais comum em países desenvolvidos); presença de isolados mais virulentos, clima, tratamento e qualidade da água que abastece as regiões, resistência a diversos antissépticos e desinfetantes e a ampla distribuição de cistos no ambiente (Ibrahim *et al.*, 2007). Dentre os fatores de riscos associados ao desenvolvimento de ceratite amebiana em usuários de lentes de contato, pode-se citar o uso de soluções salinas caseiras, a má higienização das lentes e estojos de lentes de contato, que favorece a proliferação microbiana e a formação de biofilmes na superfície das lentes, lesões na córnea e o uso por tempo prolongado da lente de contato (Siddiqui & Khan, 2012).

Atualmente ainda ocorrem subnotificações das infecções acantamebianas, principalmente pela dificuldade no diagnóstico clínico e laboratorial (Lorenzo-Morales *et al.*, 2013). Os sintomas da ceratite amebiana são muito semelhantes aos da ceratite

causada por herpes vírus e fungos e em muitos casos ocorre a identificação incorreta do agente etiológico (Claerhout *et al.*, 2004). O uso de lentes de contato associada a dor local intensa em paciente com infecção ocular é indicativo de ceratite amebiana (Siddiqui & Khan 2012). Dentre os métodos utilizados em laboratórios clínicos, o cultivo de biópsia da córnea e microscopia do raspado da córnea corados com hematoxilina-eosina ou outros corantes mais específicos, como o branco de calcofluor, que reage especificamente com as paredes celulósicas das formas de resistência de *Acanthamoeba* spp., os cistos, são amplamente utilizados (Da Rocha-Azevedo *et al.*, 2009). Para o diagnóstico de ceratite amebiana em usuários de lentes de contato, a análise também pode ser feita através da análise das lentes de contato e de seus estojos (Marciano-Cabral & Cabral 2003). A microscopia confocal *in vivo* surgiu como uma valiosa ferramenta não invasiva para o diagnóstico de ceratite infecciosa grave (Visvesvara *et al.*, 2007). Metodologias baseadas na reação em cadeia da polimerase (PCR) também foram desenvolvidas para a detecção de *Acanthamoeba* spp., já que existem sequências de reconhecimento específicas para este gênero de amebas (Lehmann *et al.*, 1998; Qvarnstrom *et al.*, 2006).

Os tratamentos utilizados para infecções por *Acanthamoeba* spp. são empíricos, baseados no uso de antimicrobianos em numerosas combinações (Lorenzo-Morales *et al.*, 2010; Roberts & Henriquez, 2010). A maioria deles tem ação sobre os trofozoítos, mas pouco efetivos sobre os cistos, o que leva à recidiva das infecções na maioria dos casos (Schuster & Visvesvara, 2004; Dart *et al.*, 2009). Dentre as drogas atualmente recomendadas para o tratamento de ceratite por *Acanthamoeba* spp. estão o polihexametileno biguanida (PHMB), clorohexidina, diamidinas, isotionato de propamidina (Brolene) e hexamidina (Desomedine). Ainda podem ser utilizados

antifúngicos como miconazol, fluconazol e cetoconazol e em casos de infecção bacteriana associada, pode-se ainda utilizar neomicina ou cloranfenicol. O tratamento da infecção ocular é difícil e de longo prazo. As drogas de aplicação tópica são utilizadas por dois ou três dias, a cada hora. Posteriormente o número de aplicações por dia é reduzido, porém o tratamento pode durar vários meses, chegando a um ano (Lorenzo-Morales *et al.*, 2010; Trabelsi *et al.*, 2012). O tratamento, quando realizado na fase tardia, poderá não ter o efeito esperado, fazendo-se necessárias intervenções cirúrgicas como ceratoplastia, debridamento e transplante de córnea (Marciano-Cabral & Cabral, 2003). Quando o tratamento não é realizado, poderá ocorrer ulceração da córnea, com presença de infiltrado no estroma, perfuração e finalmente, perda de visão (Saeed *et al.*, 2009). O ponto-chave para se evitar a ceratite amebiana é a prevenção, praticando medidas de higiene adequadas na manipulação e estocagem das lentes de contato. Apesar da grande resistência que apresentam os cistos de *Acanthamoeba* spp. (Aksozek *et al.*, 2002), existem diversos compostos amebicidas capazes de descontaminar lentes de contato e seus respectivos estojos.

2.2.3.2 Encefalite amebiana granulomatosa

A encefalite amebiana granulomatosa (EAG) é considerada uma infecção oportunista, pois quase todos os casos relatados até o momento se referem a indivíduos imunologicamente debilitados, especialmente aqueles que já sofreram algum tipo de terapia ou situação imunossupressora como alcoolismo, gravidez, quimioterapia, uso de antibióticos de amplo espectro e pacientes com AIDS (Visvesvara *et al.*, 2007). Os mecanismos associados com a patologia não estão muito claros, mas as complicações patofisiológicas envolvem o sistema nervoso central (SNC), com indução de uma

resposta pró-inflamatória, a partir da invasão pela barreira hematoencefálica e a ligação ao tecido, seguida de lesão neuronal (Schuster & Visvesvara, 2004).

O acesso dos trofozoítos ao SNC pode ocorrer por duas rotas distintas: através do neuroepitélio olfativo ou através de disseminação hematogênica. No entanto, acredita-se que em seres humanos, os trofozoítos na maioria das vezes têm acesso ao sistema nervoso central através da disseminação hematogênica proveniente dos pulmões ou de infecção cutânea (Marciano-Cabral & Cabral 2003; Khan, 2008). Especificamente, acredita-se que os trofozoítos entram no parênquima cerebral, a partir do sangue, através do endotélio capilar ou do líquido cefalorraquidiano por meio de células endoteliais do plexo coróide (Khan, 2007). Uma vez no sistema nervoso central, as células primárias do sistema imunes (microglia e macrófagos periféricos) interagem com *Acanthamoeba* spp. provocando a libertação de mediadores pró-inflamatórios (Marciano-Cabral & Cabral 2003).

EAG é caracterizada pela formação de granulomas (Figura 5), no entanto, raramente são observados granulomas em indivíduos com imunodeficiência grave (Martinez & Visvesvara, 1997). Granulomas formados durante o processo de EAG são compostos de amebas, microglia, macrofagos, célular polimorfonucleares, células T e células B (Khan, 2008). O período de incubação na EAG pode variar de semanas a meses. Após esse período, vários sintomas podem aparecer, como dor de cabeça acompanhada de febre, náuseas, vômitos, letargia, mudança da personalidade e demência (Marciano-Cabral *et al.*, 2000). O desenvolvimento da doença é fulminante e geralmente o diagnóstico só é obtido após a necropsia, por análise do fluido cérebro-espinhal ou do próprio tecido cerebral, através da análise microscópica das formas trofozoíticas, porém, a constatação de altos níveis de anticorpos *Acanthamoeba*-

específicos pode ajudar na confirmação da suspeita da infecção além de métodos baseados na reação em cadeia da polimerase (PCR) (Maclean *et al.*, 2007; Visvesvara *et al.*, 2007).

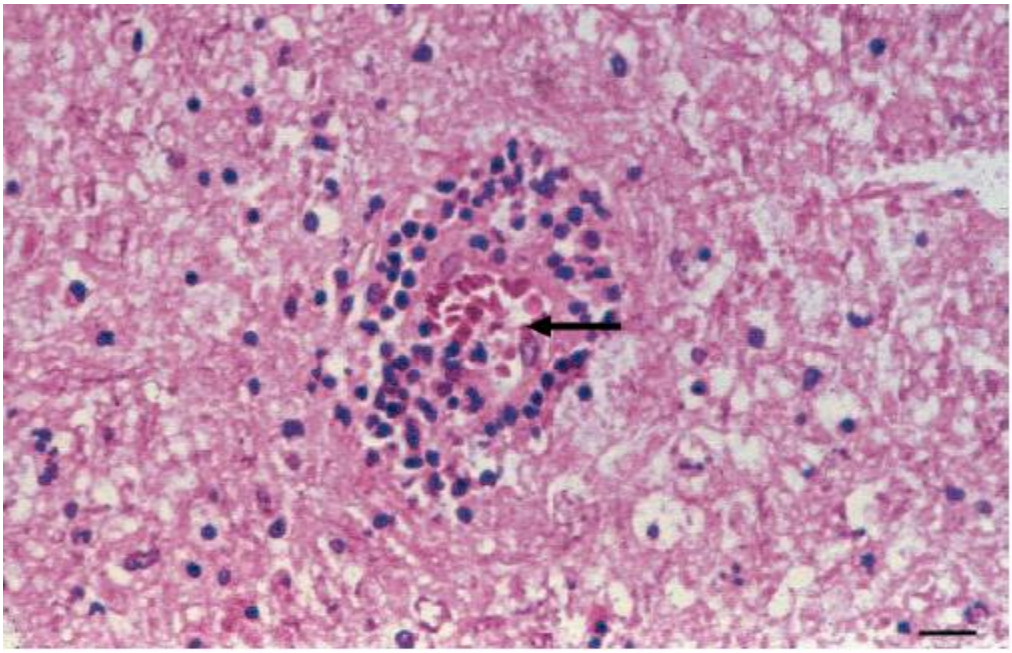

Figura 5. Granuloma característico associado com Encefalite Amebiana Granulomatosa (EAG). Coloração por hematoxilina-eosina de um granuloma com a presença de microglia e macrófagos em torno de *Acanthamoeba* spp. (indicada pela seta). Barra= 200 µm. Fonte: Marciano-Cabral & Cabral (2003).

O tratamento da EAG é extremamente problemático, especialmente pela falta de sintomas específicos e de métodos diagnósticos eficientes (Visvesvara *et al*., 2007). Normalmente os pacientes com comprometimento do SNC são tratados com um coquetel de antibióticos e antifúngicos, como por exemplo, rifampicina, trimetroprina, cetoconazol, entre outros (Marciano-Cabral & Cabral, 2003). Todavia, o sucesso no prognóstico é baixo, devido à demora do diagnóstico, ao estado imunodeficiente do paciente e à falta de drogas específicas para eliminar o patógeno.

Até o momento, aproximadamente 150 casos em todo o mundo têm sido descritos (Trabelsi *et al.*, 2012). Provavelmente estes números têm sido subestimados, visto que dados epidemiológicos referentes à EAG são raros, normalmente trata-se de uma doença secundária e também pela falta de conhecimento acerca da EAG. Na França, um único caso de infecção disseminada e fatal de EAG por *A. lenticulata* foi relatado em um paciente com transplante de coração (Barete *et al.*, 2007). A taxa aproximada de mortes associadas a encefalite amebiana granulomatosa foi sugerida de 1,57 mortes por 10.000 mortes de HIV/AIDS nos Estados Unidos e este número pode ser muito maior em países menos desenvolvidos (Siddiqui & Khan, 2012).

2.2.3.3 Outras infecções causadas por *Acanthamoeba*

Além do comprometimento da córnea e do SNC, *Acanthamoeba* spp. também podem infectar outros sistemas, de maneira também dependente do estado imunológico deprimido do hospedeiro. *Acanthamoeba* spp. são capazes de causar infecções cutâneas, sinusite, otites, pneumonites, nefrites, osteomielites, além de infecções sistêmicas (Schuster & Visvesvara, 2004).

A infecção cutânea causada por *Acanthamoeba* spp. é uma infecção oportunista rara em pacientes imunocomprometidos, em particular em pacientes infectados com HIV, e é caracterizada pela presença de nódulos eritematosos ou até ulcerações (Visvesvara *et al*., 2007; Trabelsi *et al*., 2012). Estes tipos de lesões são possíveis rotas de entrada para a disseminação e infecção do SNC, onde existe uma correlação entre acanthamebíase cutânea e disseminação de amebas para outros sítios no hospedeiro (Marciano-Cabral & Cabral, 2003). Histologicamente, as lesões cutâneas apresentam focos de necrose cercadas por células inflamatórias, vasculites e a presença

das amebas que podem estar associadas aos vasos sangüíneos (Friedland *et al.*, 1992). O tratamento da amebiase cutânea, sem envolvimento do SNC, incluí aplicações tópicas de gluconato de clorexidina e creme de cetoconazol, além de pentamidina isotionato, sulfadiazina, flucitosina, fluconazol, ou itraconazol que têm resultados terapêuticos de sucesso (Visvesvara *et al.*, 2007).

Osteomielites também têm sido associadas à presença de *Acanthamoeba* spp. em pacientes imunodeprimidos que receberam enxertos ósseos, em transplantados e diabéticos (Steinberg *et al.*, 2002).

2.2.4 Fatores de virulência relacionados à patogênese de *Acanthamoeba* spp.

A capacidade de um organismo causar dano a um hospedeiro é um evento multifatorial, que depende tanto do hospedeiro quanto do patógeno para o sucesso da infecção. Isolados de *Acanthamoeba* spp. de casos de infecção amebiana geralmente mostram maior termotolerância, taxa de crescimento, habilidade de aderir a células do hospedeiro, excreção de produtos citotóxicos e evasão da resposta imune (Lorenzo-Morales *et al.*, 2013).

Diversos mecanismos moleculares estão associados à sequência de eventos observados no processo infeccioso da ceratite amebiana, desde a adesão na superfície da córnea, destruição e invasão epitelial, extravasamento para o estroma, sua degradação e forte resposta inflamatória (Clarke & Niederkorn, 2006b). De maneira similar, a sucessão de eventos que levam à EAG e às outras infecções disseminadas, também envolvem mecanismos especiais (Khan, 2006; Khan, 2007).

A adesão de trofozoítos de *Acanthamoeba* spp. as células do hospedeiro é a primeira etapa para o estabelecimento do processo infeccioso. Rocha-Azevedo *et al.* (2009) demostraram que isolados hipervirulentos apresentavam adesão diferencial a proteínas da matriz extracelular, com preferência por Laminina-1, atribuída a uma proteína de membrana de 55 kDa não caracterizada. Estudos também têm destacado o papel da proteína ligadora de manose (MBP - *mannose binding protein*) no processo de adesão. A MBP é uma proteína transmembrana, com um extenso domínio extracelular, contendo domínios ricos em cisteína e um peptídeo sinal que reconhece resíduos de manose (Garate *et al.*, 2005). A imunogenicidade desta proteína é marcante e trabalhos tem mostrado que a imunização oral de hamsters com MBP recombinante resulta na diminuição da severidade e da duração da ceratite amebiana, possivelmente devido à produção de anticorpos do tipo IgA contra esta proteína (Garate *et al.*, 2006). Alguns trabalhos têm mostrado que isolados patogênicos apresentam maiores níveis desta proteína, quando comparados com isolados não patogênicos (Visvesvara *et al.*, 2007; Panjwani, 2010; Siddiqui & Khan, 2012).

Na patogênese da encefalite por *Acanthamoeba* spp., MBPs são responsáveis pela ligação de trofozoítos à célula alvo, que aciona uma cascata de sinalização associada a proteínas Rho e uma serino protease que atua na degradação de proteínas estruturais do cérebro, contribuindo para a progressão da lesão no SNC (Siddiqui *et al.*, 2011).

Em infecções oculares, a adesão de trofozoítos a glicoproteínas manosiladas do epitélio da córnea é mediada por uma proteína ligadora de manose de 136 kDa (Garate *et al.*, 2004; Garate *et al.*, 2005). Além de ser fundamental para o processo de adesão, a ligação entre trofozoítos de *Acanthamoeba* spp. e resíduos de manose induz a

secreção de moléculas citotóxicas, como a serino protease de 133 kDa, MIP 133 (*manose induced protein* 133). A MIP133 induz apoptose de queratócitos, de células da íris, entre outras células da retina e córnea (Clarke & Niederkorn, 2006a; b). Anticorpos produzidos contra MIP-133 foram capazes de inibir o efeito citopático de sobrenadantes de *Acanthamoeba* spp. e reduzir os sintomas da ceratite amebiana em cobaias imunizadas (Hurt *et al.*, 2003).

Algumas enzimas degradativas produzidas por *Acanthamoeba* spp. atuam como fatores que auxiliam na virulência de isolados patogênicos (Omana-Molina *et al.*, 2013). *Acanthamoeba* spp. produzem serino, cisteíno e metalo proteases, que possuem capacidade de degradar diversos substratos proteicos. No entanto, serino proteases representam a maior classe de proteases secretadas por *Acanthamoeba* spp. (Khan, 2006). Evidências experimentais indicam que serino proteases constitutivamente secretadas contribuem diretamente para o dano celular (Clarke & Niederkorn, 2006b); (Kim *et al.*, 2006). Por degradarem uma variedade de substratos proteicos (imunoglobulinas, interleucinas, elastina, fibronectina, colageno tipo I e IV) facilitam a invasão dos tecidos e a evasão da resposta imune do hospedeiro (Cho *et al.*, 2000; Alizadeh *et al.*, 2008; Ferreira *et al.*, 2009). Alguns trabalhos têm demonstrado que isolados hipervirulentos secretam maiores níveis de proteases quando comparados a isolados hipovirulentos de *Acanthamoeba* spp. (Khan *et al.*, 2000; Kim *et al.*, 2006; Rocha-Azevedo *et al.*, 2010).

Para alguns autores, a degradação do colágeno, principal proteína estrutural da córnea, resulta da ação de várias colagenases não específicas (Clarke & Niederkorn, 2006b), incluindo serino proteases, elastases, cisteíno proteases e metaloproteases. Alsam *et al.* (2005) demostraram, ao estudar uma protease de 133 kDa, quando em

contato com células que formavam um modelo *in vitro* de barreira hemato-encefálica, o aumento de 80% da permeabilidade desta barreira, revelando seu forte efeito citopático. Alguns estudos também têm demostrado que proteases desempenham importante papel no processo de encistamento e desencistamento de *Acanthamoeba* spp. (Moon, Chung *et al.*, 2008; Moon, Hong *et al.*, 2012; Lee *et al.*, 2013).

Assim como as proteases, elastases e fosfolipase A são enzimas extracelulares envolvidas na virulência de *Acanthamoeba* spp. (Siddiqui & Khan, 2012). Estas enzimas parecem estar relacionadas a EAG, capazes de degradar tecido conectivo e causar desmielinização no SNC (Mortazavi *et al.*, 2011).

Trofozoítos de *Acanthamoeba* spp. apresentam alguns mecanismos citotóxicos semelhantes aos apresentados por células NK (natural killer) e outros protozoários, como *Naegleria fowleri* e *Entamoeba histolytica*. Proteínas formadoras de poros na membrana celular do hospedeiro provocam alteração na pressão osmótica, induzindo a desorganização iônica e, consequentemente, morte celular (Michalek *et al*., 2013). Recentemente, uma toxina formadora de poro, denominada acanthaporina foi caracterizada como um importante fator de virulência em *A. culbertsoni* (Michalek *et al.*, 2013). Alguns estudos também sugerem que *Acanthamoeba* spp. são capazes de induzir morte celular programada (apoptose) nas células hospedeiras (Siddiqui & Khan, 2012), fato demonstrado pela observação de características típicas de apoptose, tais como formação de poros, formação de corpos apoptóticos, condensação da cromatina nuclear e presença de DNA fragmentado em células hospedeiras infectadas.

Outros fatores que podem contribuir para a patogênese de *Acanthamoeba* spp. incluem ecto-ATPases de diferentes pesos moleculares, que são enzimas da superfície celular capazes de hidrolisar ATP extracelular (Sissons *et al.*, 2004). Sissons

et al. (2004) demonstraram que isolados clínicos de *Acanthamoeba* spp. expressam elevados níveis de ecto-ATPase comparados a isolados ambientais. Durante a infecção por *Acanthamoeba* spp., ATP hidrolisado sob a forma de ADP atua sobre os receptores purinérgicos P2Y2 expressos na célula hospedeira, que levam à apoptose de células monocíticas (Mattana *et al.*, 2002). Além disso, a actividade de ecto-ATPase aumenta na presença de manose, o que indica que a actividade do receptor é aumentada durante a ligação à célula hospedeira (Sissons *et al.*, 2004).

Outro fator de virulência descrito para *Acanthamoeba* spp. é a enzima superóxido dismutase, que catalisa a conversão de superóxido em oxigênio e peróxido de hidrogênio e tem importante papel na defesa antioxidante. Duas enzimas superóxido dismutase foram caracterizadas em *A. castellanii* (SOD I e SOD II) (Choi *et al.*, 2000). Recentes estudos acerca das propriedades bioquímicas e funcionais da superóxido dismutase de *A. castellanii* demonstraram altos níveis de expressão da enzima sob condições de estresse oxidativo, sugerindo que esta enzima pode ter papel essencial na sobrevivência do parasito no hospedeiro por detoxificar tanto os superóxidos produzidos pelo próprio parasito endógenamente, como também pelas células de defesa do sistema imune do hospedeiro (Kim *et al.*, 2012). Estas enzimas poderão servir como alvos de drogas em infecções por *Acanthamoeba* spp.

2.2.5 Virulência: correlação *in vivo* / *in vitro*

A capacidade de isolados patogênicos de *Acanthamoeba* spp. causar infecção em cobaias vem sendo estudada desde o final da década de 1950 (Culbertson *et al.*, 1959). Desde então, camundongos, ratos, coelhos, macacos, entre outros animais

têm sido usados como objeto de estudo da patogenicidade de *Acanthamoeba* spp. (Marciano-Cabral & Cabral, 2003).

Observa-se que isolados de *Acanthamoeba* spp. ao longo de sucessivas passagens em cultura axênica apresentam atenuação da virulência, assim como a perda da capacidade de encistamento e alteração no perfil de sensibilidade a drogas (Mazur & Hadaś, 1994; Hughes *et al.*, 2003; Köhsler *et al.*, 2008). Estas alterações claramente influenciam no estudo do potencial patogênico e susceptibilidade a drogas de *Acanthamoeba* spp. (Koehsler *et al.*, 2009).

Alguns trabalhos têm demostrado que a perda de virulência de *Acanthamoeba* spp. por longo tempo de cultura *in vitro* e sucessivas passagens em cultura axênica ocorre nos primeiros seis meses, assim como a diminuição da capacidade de encistamento (Mazur & Hadaś, 1994; Koehsler *et al.*, 2008). Acredita-se que modificações epigenéticas possam ocorrer como a metilação do DNA e/ou modificações de histonas, resultando no silenciamento do gene e/ou aumento da expressão de genes relacionados (Moazed, 2001; Peterson & Laniel, 2004).

Amebas previamente inoculadas em camundongos apresentam maior capacidade de matar cobaias do que aquelas mantidas em cultivo axênico (Mazur & Hadaś, 1994). A virulência de isolados de *Acanthamoeba* spp. pode ser recuperada pela passagem seriada em ratos e re-isolamento a partir de órgãos infectados, como demonstrado no estudo realizado por Xuang *et al*. (2009). A recuperação da capacidade do isolado em infectar o hospedeiro está associada à alta regulação de genes relacionados à virulência, cujos produtos podem desempenhar um papel na patogênese de *Acanthamoeba* spp.

2.2.6 Papel do sistema imune do hospedeiro em infecções causadas por *Acanthamoeba* spp.

A exposição ambiental a *Acanthamoeba* spp. é comum e trofozoítos viáveis têm sido isolados de espécimes coletados da mucosa oral e da nasofaringe de indivíduos normais. Certamente a resposta imunológica tem um papel fundamental na proteção do hospedeiro, desde a produção de anticorpos séricos contra *Acanthamoeba* spp. em indivíduos sadios até a dependência do estado imunológico do indivíduo para o estabelecimento da EAG ou das outras infecções disseminadas (Clarke & Niederkorn, 2006). Na ceratite amebiana, tanto a resposta imune inata quanto a resposta imune adaptativa participam das defesas do hospedeiro contra *Acanthamoeba* spp. (Li e Sun, 2008).

Na resposta imune inata, neutrófilos são determinantes na resolução da ceratite amebiana através da produção, pelas células da córnea infectada, de uma proteína chamada MIP-2 (*macrophage inflamatory protein 2*), um fator quimiotático para neutrófilos (Hurt *et al.*, 2001). A importância de MIP-2 foi testada ao se inocular anticorpos anti-MIP-2 em cobaias infectadas, resultando em agravamento do quadro infeccioso (Hurt *et al*., 2001). Macrófagos também são extremamente importantes na linha de defesa contra *Acanthamoeba* spp. Depleção de macrófagos resulta numa exacerbação da ceratite amebiana em cobaias (Van Klink *et al.*, 1996). Assim como observado em neutrófilos, macrófagos ativados são capazes de destruir *Acanthamoeba* spp., sendo os principais componentes dos granulomas encontrados nos tecidos infectados (Clarke *et al*., 2006; Li & Sun, 2008). Todavia, em quadros de imunodepressão, macrófagos perdem o potencial quimiotático para *Acanthamoeba* spp.,

permitindo o estabelecimento e a piora do quadro infeccioso da EAG (Marciano-Cabral & Bradley, 2001; Marciano-Cabral *et al.*, 2006).

Com relação à resposta imune adaptativa, é notória a produção de anticorpos anti-*Acanthamoeba* em indivíduos saudáveis (Niederkorn, 2002). O papel dos anticorpos na defesa do organismo contra a ceratite amebiana ainda precisa ser elucidado. Observa-se que resposta imune adaptativa não é efetiva na prevenção de re-infecções (Clarke & Niederkorn, 2006). Anticorpos IgA, presentes nas lágrimas, são efetivos na proteção contra a adesão de *Acanthamoeba* spp. às células da córnea (Clarke & Niederkorn, 2006). Contudo, em pacientes infectados, o nível desta imunoglobulina cai drasticamente, indicando que algum mecanismo regule a produção deste anticorpo (Alizadeh *et al.*, 2001). Com relação à IgG, um aumento foi observado em pacientes com ceratite (Alizadeh *et al*., 2001).

2.2.7 Estudos proteômicos de *Acanthamoeba* spp.

A proteômica tem como objetivo estudar as propriedades das proteínas, seus níveis de expressão, suas funções, modificações pós-traducionais, interações entre proteínas e mecanismos regulatórios (Blackstock & Weir, 1999). A descrição do proteoma de um organismo, além de fornecer um catálogo do conjunto de proteínas que está sendo expresso pelo genoma, também fornece dados de expressão celular sob condições definidas e a distribuição dessas proteínas na célula (Cash, 1998). Diferentes técnicas proteômicas têm sido utilizadas na investigação de diversos aspectos da biologia, bioquímica e fisiologia de diversos protozoários (Tolstrup *et al.*, 2007; Bille *et al.*, 2009; Wen & Garg, 2012; Ali *et al.*, 2012; Jerlstrom-Hultqvist *et al.*, 2012; Pawar *et al.*, 2012; Urbaniak *et al.*, 2012). Essas análises têm revelado a diversidade de proteínas

expressas por diferentes parasitos, auxiliando na elucidação de mecanismos moleculares utilizados por cada um deles na interação com espécies hospedeiras, na manutenção da infecção e na identificação de biomarcadores potenciais para o diagnóstico, alvos vacinais e o desenvolvimento de novas drogas.

Para a análise de proteoma, dois princípios são utilizados: o de separação e o de identificação de proteínas. As duas técnicas de separação mais utilizadas são a eletroforese bidimensional e a cromatografia multidimensional. Na eletroforese bidimensional, as proteínas são separadas por uma focalização isoelétrica (IEF) e posteriormente, por massa molecular (SDS-PAGE). Essa estratégia possibilita alta resolução para separar proteínas de um extrato proteíco complexo (Joubert *et al.*, 2000). Na cromatografia multidimensional, as proteínas são separadas pela utilização de cromatografia em várias colunas, com princípios de separação distintos geralmente empregando-se a cromatografia em colunas de troca iônica e fase reversa (Maynard *et al.*, 2004).

Técnicas como eletroforese bidimensional (2-DE) e cromatografia líquida acoplada a espectrometria de massas em tandem (LC-MS/MS) podem ser utilizadas na análise e comparação de diferentes formas de vida parasitárias, na comparação de isolados virulentos e avirulentos, identificando diferenças na expressão e modificação pós-traducional de proteínas (Regidor-Cerrillo *et al.*, 2012; Walker *et al.*, 2012; Wang *et al.*, 2013). Estudos imunoproteômicos, utilizando técnicas proteômicas associadas a análises imunológicas, têm auxiliado na identificação de proteínas antigênicas, com potencial imunodiagnóstico e/ou vacinal contra diferentes parasitos (Marcilla *et al.*, 2008; Morassutti *et al.*, 2012).

Para *Acanthamoeba* spp. poucos estudos proteômicos foram descritos, dentre eles apenas abordagens relacionadas a investigação de proteínas expressas durante a fase de encistamento (Bouyer *et al.*, 2009; Leitsch *et al.*, 2010). Nenhuma análise proteômica do repertório de proteínas expressas por trofozoítos de *Acanthamoeba* spp. foi realizada até agora. Portanto, uma análise do repertório de proteínas expressas por formas invasivas de *A. polyphaga* e a comparação de isolados virulento e avirulento poderá contribuir para um melhor entendimento da biologia do parasito, com a elucidação de aspectos relacionados aos mecanismos de controle do seu ciclo celular e à virulência, e para a identificação de antígenos diagnósticos e proteínas-alvo para terapia.

Para abordagens experimentais de proteômica é necessária uma base de dados de sequências gênicas abrangente. Para estudos proteômicos de *Acanthamoeba* spp., estão disponíveis sequências nucleotídicas e proteicas depositadas no GenBank e bancos de ESTs de *A. castellanii* e *A. healyi* (Kong *et al.*, 2001; Anderson *et al.*, 2005; Moon *et al.*, 2009). Recentemente o genoma completo de *A. castellanii* foi sequenciado, possibilitando também a construção de bancos de dados de sequências ortólogas, capazes de maximizar o processo de identificação de proteínas de *A. polyphaga* (Clarke *et al.*, 2013).

3. RESULTADOS E DISCUSSÃO

Os resultados da presente tese estão organizados em três capítulos apresentados na forma de artigos científicos.

No capítulo 1 é apresentado o artigo intitulado "*Acanthamoeba* T3, T4 and T5 in swimming-pool waters from Southern Brazil", publicado na revista *Acta tropica*. Este artigo descreve a caracterização genotípica de isolados ambientais de *Acanthamoeba* spp. obtidos de água de piscinas de clubes da cidade de Porto Alegre – RS e representa o primeiro trabalho de genotipagem de isolados ambientais do grupo de pesquisa de AVL da UFRGS. A partir deste trabalho, a caracterização genotípica de outros isolados clínicos e ambientais foram realizados no laboratório e publicados em colaboração durante o período do doutorado e são apresentados no apêndice, ao final da presente tese.

No capítulo 2 é apresentado o artigo intitulado "Proteomic profiling of the infective trophozoite stage of *Acanthamoeba polyphaga*", submetido para publicação na revista *Acta tropica*. Este artigo descreve o primeiro estudo proteômico de *A. polyphaga* e estabelece uma análise abrangente das proteínas expressas por trofozoítos.

O capítulo 3 apresenta o manuscrito em preparação, que tem como título "Comparative proteomic analysis of *Acanthamoeba polyphaga* attenuated isolate with enhanced virulence after experimental infection of rat and identification of antigenic proteins". Neste trabalho foi realizada uma análise proteômica comparativa de um isolado ambiental de *A. polyphaga* sob duas condições: com virulência atenuada por longo tempo de cultivo *in vitro* e com ativação da virulência pós-passagem em modelo animal. Várias proteínas diferencialmente expressas foram identificadas possivelmente envolvidas em mecanismos de patogenicidade da ameba.

CAPÍTULO I

Manuscrito publicado na revista "*Acta Tropica*"

Acanthamoeba T3, T4 and T5 in swimming-pool waters from Southern Brazil

Karin Caumo, Marilise B. Rott

Research highlights

▸ Species of *Acanthamoeba*, an opportunistic pathogen, are frequently isolate of water sources. ▸ *Acanthamoeba* T3, T4 and T5 were isolated in swimming-pool water from Southern Brazil. ▸ The genotype T5 of *Acanthamoeba* is a common contaminant in swimming-pool water

Graphical abstract

			Reference sequences*	
Isolate	**GenBank Accession**	**Genotype**	**GenBank accession**	**Identity (%)**
PO1	HM055894	T5	AB525818	95
PA1	HM055892	T5	GU597014	96
PA2	HM055896	T5	GU573877	95
PI2	HM055885	T5	EF176004	95
PH2	HM055887	T5	GU573877	96
PI3	HM055889	T5	GU573877	96
PI4	HM055890	T4	AY702990	97
PO5	HM055891	T5	AB327060	96
PT5	HM055893	T4	EU934064	96
PI6	HM055895	T5	EF176004	95
PM6	HM055897	T5	GU573877	96
PI8	HM055888	T4	EU934065	97
P7	HM055886	T3	FJ042644	97

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Acanthamoeba T3, T4 and T5 in swimming-pool waters from Southern Brazil

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ABSTRACT

Species of *Acanthamoeba*, known to cause keratitis (AK) and granulomatous encephalitis in humans are frequently isolated from a variety of water sources. In this study, 13 *Acanthamoeba* isolates from swimming pools were classified at the genotype level based on the sequence analysis of the *Acanthamoeba* small-subunit rRNA gene. Nine of the 13 isolates were genotype T5, three were genotype T4, and one was T3. Several genotypes have been reported worldwide as causative agents of AK, including genotypes T3, T4, and T5. The present study indicates that genotype T5 is a common contaminant in swimming-pool water.

1. Introduction

Acanthamoeba spp. are free-living amoebae (FLA) and are among the most abundant protozoa in the environment. Several species of *Acanthamoeba* are clinically significant, with the potential to cause a corneal infection termed *Acanthamoeba* keratitis (AK); some strains can cause granulomatous amoebic encephalitis (GAE), a fatal brain infection (Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004; Khan, 2006; Visvesvara et al., 2007).

Human amoebic keratitis infection occurs through contamination from rinsing contact lenses in non-sterile water, or through wearing contact lenses while bathing or swimming in a non-disinfected aquatic environment (Martínez and Visvesvara, 1997; Radford et al., 1998; Sharma et al., 2000; Parija et al., 2001; Khan and Tareen, 2003; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004).

At the generic level, *Acanthamoeba* can be easily identified by its morphological characteristics. Pussard and Pons (1977) divided the genus into 3 groups according to the cyst size and other morphological features. Molecular analyses, using nuclear and mitochondrial small-subunit (SSU) rRNA genes, support the morphological group structure of the genus (Kilvington et al., 1991; Stothard et al., 1998; Booton et al., 2005). However, the division of *Acanthamoeba* isolates into different species is inconsistent (Stothard et al., 1998; Alves et al., 2000).

The taxonomy and classification of these protozoa are continually under revision, following the successful application of molecular techniques (Stothard et al., 1998; Booton et al., 2002, 2005). Evolutionary studies have led to the identification of at least 16 genotypes (T1–T16) based on rRNA gene sequencing (Stothard et al., 1998; Gast, 2001; Booton et al., 2005; Maghsood et al., 2005; Corsaro and Venditti, 2010).

Human infections by *Acanthamoeba* remain under-investigated in Brazil, however some cases of keratitis and studies of clinical and environmental isolates have been made (Alves et al., 2000; Caumo et al., 2009; Magliano et al., 2009; Carlesso et al., 2010). The genotypes of *Acanthamoeba* present in water sources associated with human activity, and the risk to human health posed by exposure to these organisms, have yet to be elucidated. In this study, 13 isolates of *Acanthamoeba* collected from swimming-pool waters in Southern Brazil were cultured, in order to discriminate the genotypes of the isolates and to analyze the correlation with strains described from other areas.

2. Materials and methods

2.1. Organisms, growth conditions, and DNA extraction

Thirteen cultures containing *Acanthamoeba* isolates obtained from swimming-pool water (Caumo et al., 2009) were used in this study. The isolates were cultured axenically in PYG medium [0.75% (wt/vol) proteose peptone, 0.75% (wt/vol) yeast extract, and 1.5%

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(wt/vol) glucose] at 30 °C. The total DNA was extracted from each isolate (containing 10^6 trophozoites/mL) according to the method of Salah and Iciar (1997).

2.2. Molecular identification of Acanthamoeba isolates

Primers JDP1 and JDP2 were used to amplify the ASA.S1 region of the gene (Rns) coding for the amoeba's nuclear small-subunit ribosomal RNA (Schroeder et al., 2001). The polymerase chain reaction (PCR) was performed as described by Booton et al. (2004). Briefly, the amplifications were carried out in a 50 μl volume containing 20–30 ng of DNA, 0.2 mM dNTPs (Mix, Invitrogen™), 0.4 μM of each oligonucleotide, reaction buffer (50 mM KCl_2, 10 mM Tris–HCl), 1.5 mM $MgCl_2$, and 1 U of Platinum® Taq DNA Polymerase (Invitrogen™). The amplification reaction was carried out in a PTC-150 Minicycler MJ Research thermocycler, under the following conditions: 94 °C for 1 min, 67 °C for 1 min, 72 °C for 1 min, and 72 °C for 5 min. The amplification product was separated in 1.5% agarose gel, stained with 0.5 μM/ml ethidium bromide, and observed under a UV-light transilluminator.

PCR products were purified using a QIAquick® extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions, and resolved with a MegaBace 1000 automated sequencer.

In order to classify the *Acanthamoeba* isolates of the present study, 13 sequences of 18S rDNA gene were uploaded into the Basic Local Alignment Search Tool (BLAST) program of the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to search for the most similar sequences.

2.3. GenBank accession numbers

The 18S rRNA gene sequences of the 13 *Acanthamoeba* isolates determined in this study were deposited in the GenBank database under accession numbers HM055885 to HM055897.

3. Results and discussion

Of the 65 samples of swimming-pool water, 13 (20%) were positive for FLA, and all of the amoebae detected were identified by morphology as belonging to the genus *Acanthamoeba* (Caumo et al., 2009). As part of a more comprehensive study examining the genotype of *Acanthamoeba* spp. isolates obtained from swimming-pool water in social clubs in Porto Alegre, we identified *Acanthamoeba* genotypes molecularly, using 18S rDNA sequencing of 13 isolates. Amplification of genus-specific amplimers ASA.S1 with primers JDP1 and JDP2 was successful in all 13 isolates tested. Amplimers ASA.S1 (approximately 500 bp) were obtained from all isolates.

The 13 18S rRNA sequences from the *Acanthamoeba* isolates were aligned with *Acanthamoeba* sequences retrieved from GenBank. Our results revealed that *Acanthamoeba* isolates from swimming pools belong to genotypes T3, T4, and T5 (Table 1).

Nine of the 13 sequences revealed a strict correspondence with the available sequences of the T5 genotype. The percentage of identity between the obtained sequences and the nine T5 sequences chosen as reference ranged from 95% to 96%. Three isolates are closely related to *Acanthamoeba* genotype T4 sequences chosen as references, ranged from 96% to 97% identity. One of the 13 isolates analyzed (P7) showed a strict correspondence with the deposited sequences for genotype T3, with 97% identity with the T3 sequence chosen as reference.

The three genotypes of *Acanthamoeba* identified in this study are commonly found in clinical isolates and in the environment (soil and water), as reported in other studies. Several studies worldwide have assayed tap and spring water systems, rivers, and swimming pools in order to determine the abundance of these amoebae in aquatic environments related to human habitats, which is a key predisposing factor in *Acanthamoeba* infections (Ma et al., 1990; De Jonckheere, 1991; Kilvington and White, 1994; Radford et al., 1998; Booton et al., 2002; Khan and Paget, 2002; Ettinger et al., 2003; Seal, 2003).

Table 1
Genotypic characterization of isolates found in swimming pools in Porto Alegre-RS.

Isolate	GenBank accession	Genotype	Reference sequences[a]	
			GenBank accession	Identity (%)
PO1	HM055894	T5	AB525818	95
PA1	HM055892	T5	GU597014	96
PA2	HM055896	T5	GU573877	95
PI2	HM055885	T5	EF176004	95
PH2	HM055887	T5	GU573877	96
PI3	HM055889	T5	GU573877	96
PI4	HM055890	T4	AY702990	97
PO5	HM055891	T5	AB327060	96
PT5	HM055893	T4	EU934064	96
PI6	HM055895	T5	EF176004	95
PM6	HM055897	T5	GU573877	96
PI8	HM055888	T4	EU934065	97
P7	HM055886	T3	FJ042644	97

[a] Percentage if identity and access number compared to GenBank sequences of isolates of the present work.

This study of the distribution of *Acanthamoeba* genotypes in water samples from swimming pools in Brazil, indicated genotype T5 as the most common among the sequences analyzed. This is the second most abundant clade found in the general environment, and is underrepresented in AK cases (Booton et al., 2005; Ledee et al., 2009).

Isolates T5 genotype are usually free-living, although there are cases where strain belonging to this genotype has been isolated from patients with *Acanthamoeba* keratitis (Spanakos et al., 2006) and disseminated acanthamoebiasis (Barete et al., 2007). The presence of genotype T5 strains has been described as the main factor responsible for *Acanthamoeba* keratitis infections in the United States (Ledee et al., 2009). The T4 and T5 genotypes have also been isolated previously from samples from both environmental (soil and water) and contact-lens storage cases in the Philippines, where genotype T5 was more commonly isolated than was T4 (Rivera and Adao, 2008, 2009).

Amoebic keratitis is a water-related syndrome caused by *Acanthamoeba*, and the T4 genotype is the most commonly reported among keratitis isolates (Marciano-Cabral and Cabral, 2003; Booton et al., 2005). This genotype is also the most common in environmental samples (Booton et al., 2004, 2005). Isolates PI4, PT5, and PI8 were classified as T4.

The T3 genotype is less prevalent in the environment than T4 or T5 (Ledee et al., 2009). According to Edagawa et al. (2009), this genotype is generally considered to be non-pathogenic. Since the first case of *A. griffin*-associated AK in the UK (Ledee et al., 1996), very few cases caused by T3 have been reported (Stothard et al., 1998; Zhang et al., 2004; Maghsood et al., 2005; Booton et al., 2009; Nagyová et al., 2010).

In our study, we found potentially pathogenic *Acanthamoeba* genotypes in swimming-pool water, representing health risks in aquatic sources. Our results confirm that genotyping of isolates from different environments is essential for describing the global distribution of sequence types as well as the frequency of genotypes related to AK. Further tests are necessary to fully determine the pathogenicity of these isolates, in order to evaluate the risks posed by *Acanthamoeba* to users of swimming pools.

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CAPÍTULO II

Manuscrito submetido à revista "*Acta Tropica*" no dia 22 de fevereiro de 2013.

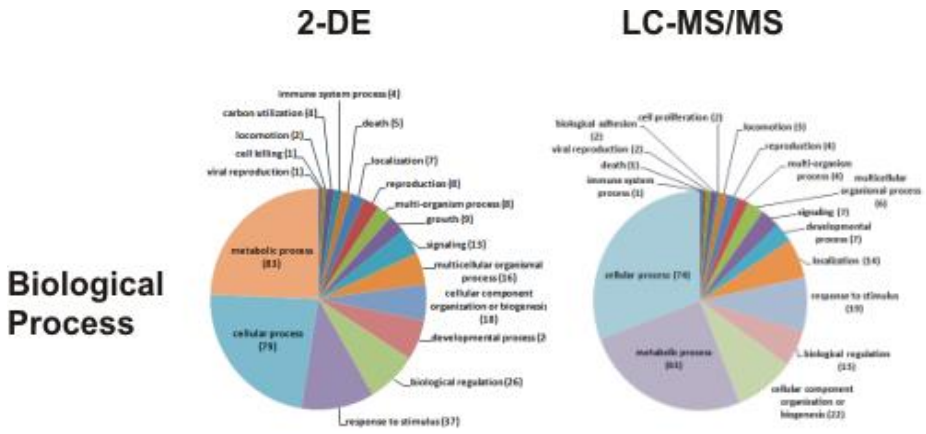
Proteomic profiling of the infective trophozoite stage of *Acanthamoeba polyphaga*

Karin Caumo, Karina M. Monteiro, Thiely Ott, Glauber Wagner, Henrique B. Ferreira, Marilise B. Rott

Research highlights

We established a comprehensive analysis of the proteins expressed by infective trophozoite stage of *Acanthamoeba polyphaga* based on complementary 2-DE-MS/MS and gel-free LC-MS/MS approaches.

Graphical abstract



Proteomic profiling of the infective trophozoite stage of *Acanthamoeba polyphaga*

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ABSTRACT

Acanthamoeba polyphaga is a free-living protist pathogen, capable of causing a blinding keratitis and fatal granulomatous encephalitis. Trophozoite is the form infective of *Acanthamoeba* spp. and can provoke infections in a variety of mammalian hosts and humans, as result of complex interaction host-parasite. The damage caused by trophozoites in human corneal or brain infections is the result of several different pathogenic mechanisms not elucidated at the molecular level so far. This work established a comprehensive analysis of the proteins expressed by *A. polyphaga* (ATCC 30872) trophozoites based on complementary 2-DE MS/MS and gel-free LC-MS/MS approaches. Overall, 192 nonredundant proteins were identified. An *A. polyphaga* proteomic map in pH range 3-10 was produced, with protein identification for 136 out of 370 resolved spots. Functional classification of identified proteins revealed several proteins with potential relevance for parasite survival and infection of mammal hosts, including surface proteins and proteins related to defense mechanisms. This study describes the first comprehensive proteomic survey of the trophozoite infective stage of an *Acanthamoeba* species, and provides foundations to prospective, comparative and functional studies of *Acanthamoeba* proteins involved in molecular mechanisms of survival, development, and pathogenicity.

Keywords: *Acanthamoeba* infective stage; 2-DE proteomic map; gel-free LC-MS/MS; global protein analysis; pathogen-host interaction.

1. Introduction

Free-living amoebae (FLA) belonging to genus *Acanthamoeba* are protozoans ubiquitously distributed in nature, and are adapted to live in a wide variety of natural and man-made environments (Schuster and Visvesvara, 2004; Caumo et al., 2009; Magliano et al., 2009; Carlesso et al., 2010; Winck et al., 2011; Siddiqui and Khan, 2012a). *Acanthamoeba* spp. have gained increasing attention from the scientific community over the years, due to their versatile roles in the ecosystem. The active trophozoite stage, which exhibits vegetative growth and is the infective form, feeds on bacteria, algae, and yeast. Besides, it is also a reservoir for pathogenic microorganisms resistant to phagocytosis by the amoebae, and these interactions may help transmit microbial endosymbionts to the susceptible hosts and/ or endossymbionts may contribute to the pathogenicity of *Acanthamoeba* spp. (Visvesvara et al., 2007; Siddiqui and Khan, 2012b).

Acanthamoeba spp. have also increased in medical importance during the last decade due to their potential to infect human hosts, causing severe diseases, such as granulomatous amoebic encephalitis (GAE), a chronic brain infection that occurs more frequently in immunosuppressed individuals; amoebic keratitis (AK), a sight-threatening infection of the cornea that is related to contact lens misuse; and disseminated infections (Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007). Of the approximately 20 species of *Acanthamoeba* that have been identified, only twelve, among then *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, *Acanthamoeba astronyxis*, *Acanthamoeba culbertsoni and Acanthamoeba healyi*, have been linked to human disease (Corsaro and Venditti, 2010; Visvesvara, 2010).

Acanthamoeba spp. trophozoites also have been used extensively as model systems to study eukaryotic cell biology, due to their relatively large size, rapid growth in culture, and active motility (Klopocka et al., 2009; Chrisman et al., 2010; Brzeska et al., 2012; Siddiqui and Khan, 2012c). The well-developed cytoskeleton of these organisms turned them specially good models for the understanding of actin cytoskeleton-based motility and other aspects of the molecular biology of cell motility (Siddiqui and Khan, 2012a).

Proteomic studies have been described for many protozoa, including *Entamoeba histolytica*, *Giardia intestinalis* and *Leishmania donovani* (Tolstrup et al., 2007; Biller et al., 2009; Ali et al., 2012; Jerlstrom-Hultqvist et al., 2012; Pawar et al., 2012a). These analyzes have revealed a diversity of proteins expressed by different parasitic species, helping to elucidate the molecular mechanisms used for interaction with host species and to identify potential biomarkers for diagnosis and targets for the development of new drugs or vaccines. For *Acanthamoeba* spp. few proteomic studies have been conducted, being limited to the investigation of protein expression during the encystment (Bouyer et al., 2009; Leitsch et al., 2010). Although important for a better understanding of the biology of invasive forms, the repertoire of proteins expressed by *Acanthamoeba* spp. trophozoites has been not investigated so far.

The analysis of the repertoire of proteins expressed by *Acanthamoeba* spp. trophozoites may contribute to the elucidation of the mechanisms of virulence, and for the identification of diagnostic antigens and target proteins for therapy. In this work, we established the conditions for the two-dimensional gel electrophoresis (2-DE) analysis of *A. polyphaga* trophozoites and identified resolved proteins in order to provide a

reference proteomic map. A comprehensive gel-free LC-MS/MS analyses was also performed and, overall, 192 nonredundant proteins were identified, including 136 proteins identified of 370 spots mapped in the 2-DE gel. Several groups of proteins with important roles were identified; including those metabolism-related, cytoskeleton, post translation modification, protein turnover and chaperones, proteins localized on the surface and related to defense mechanisms.The importance of the identified repertoire of trofozoite proteins for the biology of *A. polyphaga* is discussed.

2. Material and methods

2.1 *A. polyphaga* strain, cultivation, and cell protein extracts

A. polyphaga trophozoites of to the T4 genotype were obtained from the American Type Culture Collection (ATCC 30872). Trophozoites were cultured axenically in peptone-yeast-glucose (PYG) medium, as described previously (Schuster, 2002).

For protein extraction, three identical and independent cultures (biological replicates) from approximately $1x10^8$ cells (trophozoites at log growth phase) were used. Cells were harvested at 2000 x *g* for 10 min and washed twice in phosphate-buffered saline (PBS) buffer (pH 7.2), prior to resuspension in 1 ml of 25 mM Tris–HCl, pH 7.2, containing a cocktail of protease inhibitors. Cell suspensions were then lysed by sonication (25 Hz in a VC601 Sonics and Materials Inc. sonicator) in an ice bath by five 30 s cycles with 1 min interval between pulses. Lysates were centrifuged (18,000 x *g*, 15 min, 4 °C) to separate soluble and insoluble protein fractions. Soluble

proteins were quantified using a Qubit™ quantitation fluorometer and Quant-it™ reagents (Invitrogen, USA).

2.2 Two-dimensional gel electrophoresis and gel image analysis

Protein samples (2 mg) were precipitated overnight at -20 °C with two volumes of ice cold 20% (w/v) trichloroacetic acid/acetone. Protein precipitates were recovered by centrifugation (10 min at 18,000 x *g*) and washed five times with ice cold acetone. The pellet was air-dried and solubilized in 350 µL isoelectric focusing (IEF) buffer containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT), and 0.2% (v/v) ampholytes pH 3-10 (Bio-Rad, Hercules, US). The 17 cm immobilised pH gradient (IPG) strips (pH 3-10 or 4-7, Bio-Rad) were passively rehydrated with the cell extract sample in IEF buffer for 16 h and IEF was performed in a Protean IEF cell system (Bio-Rad, Hercules, US) with up to 50,000 VH at a maximum voltage of 10,000 V. Strips were equilibrated for 15 min in equilibration buffer I (30%, v/v, glycerol, 6 M urea, 1% DTT, a trace of bromophenol blue) and for 15 min in equilibration buffer II (equilibration solution I with DTT replaced by 4% iodoacetamide). In the second dimension, IPG strips were run vertically onto SDS-PAGE 12% gels using PROTEAN® II xi 2D Cell (Bio-Rad, Hercules, US). For each protein sample, three independent gels were run (technical replicates). Gels were stained with 0.1% Coomassie Brilliant Blue G (Acros, Geel, Belgium), scanned with a computer-assisted G-800 densitometer (Bio-Rad, Hercules, US) and analyzed with the PDQuest Basic-8.0 software (Bio-Rad), followed by additional visual analysis. To determine experimental p*I* and Mw coordinates for each single spot, 2-DE gels were calibrated using a select set of reliable identification landmarks distributed throughout the entire gel.

2.3 Sample preparation for mass spectrometry

Protein spots were manually excised from Coomassie stained 2-DE gels and in-gel digested with trypsin. Gel plugs were treated in three washing steps with 180 µl of 50% acetonitrile (ACN) and 50 mM ammonium bicarbonate for 15 min, followed by one washing step with 180 µl of acetonitrile. After the washing procedures, gel plugs were dried by vacuum centrifugation and digested for 18-24 h at 37 °C using 12 µl of 10 mg ml^{-1} modified porcine trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega), diluted to 25 mM in NH_4HCO_3. After tryptic digestion, peptides were extracted in two washing steps with 50 ml of 50% acetonitrile and trifluoroacetic acid (TFA) for 1 h. Extracted peptides were dried and resuspended in 10 µl of 0.1% TFA.

For gel free LC-ESI-Q-TOF MS/MS (LC-MS/MS) experiments, protein extracts were prepared from three identical and independent cultures (biological replicates). Protein samples were diluted in denaturing buffer (25 mM NH_4HCO_3/ 8 M urea, pH 8.9), reduced by adding DTT (0.02 µg/µg protein), and carboxyamidomethylated with iodoacetamide (0.1 µg/µg protein). Samples were further diluted with 25 mM NH_4HCO_3 to a urea final concentration of 1 M and trypsin was added at a ratio of 0.01 µg/µg protein. After digestion for 4 h at 37 °C, an additional aliquot of enzyme was added, and samples were further incubated for 16-20 h at 37 °C. The resulting peptides were desalted using OASIS® HLB Cartridge (Waters, USA) and eluted in 300 µL of 70% ACN/0.1% TFA.

2.4 Mass spectrometry analyses

Peptides from digested protein spots were analyzed by on-line liquid chromatography/mass spectrometry (LC-MS/MS) using a Waters nanoACQUITY UPLC system coupled to a Waters Micromass Q-TOF Micro or Q-TOF Ultima API mass spectrometer (Waters MS Technologies, UK). The peptides were eluted from the reverse-phase column toward the mass spectrometer at a flow rate of 200 nl/min with a 10-50% water/ACN 0.1% formic acid linear gradient over 10 min for peptides obtained of protein spots and 45 min for peptides of protein extracts. Analyses were performed using the data-dependent acquisition (DDA) mode where each full MS scan was followed by three consecutive MS/MS scans. For each survey scan, the three most intense multiple charged ions over a threshold of 8 counts were selected for MS/MS analysis. The collision energies for peptide fragmentation were set using the charge state recognition files for +2, +3, and +4 peptide ions provided by MassLynx (Waters). MS/MS raw data were processed using Protein-Lynx Global Server 2.0 software (Waters), and peak lists were exported in the micromass (.pkl) format. For experiments with protein extracts, at least two independent LC-MS/MS runs were performed.

2.5 Database searching and bioinformatics analyses

For peptide identification, all MS/MS data were searched against databases using MASCOT software 2.0 (http://www.matrixscience.com, Matrix Science) with the following parameters: a maximum of one missed cleavage site, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine and a 0.1 mass unit tolerance on parent and fragment ions. The significance threshold was set at $p < 0.05$, and only peptides with individual ion scores above this significance threshold were considered for protein identification. The MS/MS spectra of protein identifications based on a

single peptide and on borderline scores were manually inspected for acceptance. In addition, a decoy database search was used to estimate false discovery rates for LC-MS/MS analyses, resulting in average probabilities of 1,26% in searches against *Acanthamoeba* spp. ESTs decoy sequences, respectively.

A local *Acanthamoeba* spp. database was constructed based on the *A. castellanii* and *A. healyi* EST sequences available at GenBank dbEST (http://www.ncbi.nlm.nih.gov/dbEST/) (Jan/2011); ii) 13,770 trophozoites EST sequences of *A. castellanii* available at TBestDB (http://tbestdb.bcm.umontreal.ca) expressed of in normal conditions or genes silenced (O'Brien et al., 2007); and iii) 3,897 ESTs from *Acanthamoeba* EST DB database (http://www.amoeba.or.kr), expressed by amebae under long term *in vitro* culture, mouse brain passage or encystation derived (Moon et al., 2009).

Gene ontology (GO) terms were applied to the identified proteins using Blast2Go (Gotz et al., 2008), where Blast and annotations were performed with default parameters. Blast2Go was also used to generate the pie charts of GO terms from molecular functions, biological processes and cellular components. Eukaryotic Orthologous Group (KOG) annotations (Tatusov et al., 2003) were also assigned based on sequence similarity searches against the KOG annotated proteins (http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html).

The reference 2-DE gel map was submitted to the World-2DPAGE Repository (Submission ID: 2013.2.8--10729; http://world-2dpage.expasy.org).

3. Results

3.1. Two-dimensional electrophoresis proteomic mapping of *A. polyphaga* trophozoites

In order to resolve the prominent proteins of *A. polyphaga*, we performed 2-DE in pH range 3-10 and 4-7 IPG strips from protein extracts from amebae trophozoites under long term *in vitro* culture in standard conditions. As technical controls, all protein preparations and subsequent 2-DE were repeated three times and images representative 2-DE gels in pH range 3-10 were selected for constructing the 2-DE reference map of *A. polyphaga* trophozoite proteins (Fig. 1). PDQuest™ software was used for the image analysis of representative 2-DE gels obtained from nine independent experiments. The 2-DE protein spot profiles were highly reproducible (~90% matching between replicates), in terms of both the total number of protein spots and their relative positions and intensities. About 370 prominent proteins spots were resolved on Coomassie stained 2-DE gels, with molecular weights ranging from 18.8 to 186.7 kDa.

Prominent protein spots resolved by 2-DE (pH 3-10) from *A. polyphaga* trophozoites were submitted to ESI-Q-TOF MS/MS analysis for protein identification and 2-DE mapping. MS identification was obtained for 136 of the 370 analyzed protein spots (Table 1 and Supplementary Table A1). Seventy-six of the 101 unique proteins were identified from a single spot, while 25 of the identified proteins were represented by two or more distinct spots on the gel. The most these spots showed migrating with similar apparent molecular mass and varying p*I* from trophozoites proteome analysed suggesting post-translational modifications. Furthermore, several proteins represented in two or more distinct spots on the gel had variance in the experimental Mw indicates possible cleavage proteolytic. Spots with protein identification are indicated in Figure 1

and those possibly corresponding to possible post-translational modified proteins are indicated in Table 1 and Supplementary Table A1 in bold.

3.2 Gel free ESI-Q-TOF MS/MS analysis of *A. polyphaga* trophozoite protein extract

The gel-free analysis of the *A. polyphaga* trophozoite soluble protein extract by LC-MS/MS allowed the identification of a total of 114 unique proteins (Supplementary Table A2). Each sample was parallel analyzed two times to each biological replicate ensure data reproducibility, which was ~96% observed in these runs.

Overall, the 2-DE/ESI-Q-TOF MS/MS and gel-free LC-MS/MS complementary proteomic approaches allowed the identification of 192 non-redundant *A. polyphaga* trophozoite proteins. Just 23 of these proteins were identified by both techniques, showing that these approaches generate essentially complementary data sets (Fig. 2).

3.3 Functional analysis of the identified proteins

The functional annotation of proteins identified in protein extracts from *A. polyphaga* trophozoites was based on GO and KOG classifications. GO terms were assigned to the identified proteins on the basis of similarity using Blast2Go. In this analysis, ≥1 GO terms were assigned for 118 ESTs sequences obtained of 136 protein identification for spots analyzed (87%) and 114 ESTs sequences obtained of 114 protein identification for LS-MS/MS analysis (100%). These annotated proteins were assigned to molecular function, biological process and cellular component GO analysis (Fig. 3).

The identified proteins were grouped into 20 categories according to biological process. Metabolic process (GO:0008152), cellular process (GO:0009987), biological

regulation (GO:0065007), response to stimulus (GO:0050896) and cellular component organization or biogenesis (GO:0071840) were the most abundant process, followed by developmental process (GO:0032502), localization (GO:0051179), multicellular organismal process (GO:0032501), signaling (GO:0023052), reproduction (GO:0000003), growth (GO:0040007), multi-organism process (GO:0051704), death (GO:0016265), locomotion (GO:0040011) and immune system process (GO:0002376). Other process were identified in a small proportion of cases.

Catalytic activity (GO:0003824) and binding (GO:0005488) were the predominant molecular function categories. Other common molecular function categories included structural molecule activity (GO:0005198), transporter activity (GO:0005215), antioxidant activity (GO:0016209), enzyme regulator activity (GO:0030234), electron carrier (GO:0009055), receptor activity (GO:0004872) and others function categories were assigned with a lesser extent.

Proteins were classified into nine groups based on their corresponding cell component. The most populated GO terms involved cells (GO:0005623) and organelles (GO:0043226), followed by macromolecular complexes (GO:0032991), membrane (GO:0016020) and membrane-enclosed lumens (GO:0031974). A small fraction of protein was localized to the cell junction (GO:0030054), extracellular region (GO:0005576), symplast (GO:0055044) and synapse (GO:0045202), respectively.

According to the KOG functional classification (Fig. 2), the identified proteins of *A. polyphaga* trophozoites for 2-DE analysis and LC-MS/MS could be classified in 19 categories. These results, most of the identified proteins are related to energy production and conversion (C - 15% and 13%), posttranslational modification, protein

turnover, chaperones (O - 19% and 7%), carbohydrate transport and metabolism (G - 11% and 2%), cytoskeleton (Z - 9% and 13%), translation (J - 6% and 31%) and general function prediction only (R - 5% and 3%) categories. We also identified several proteins related to transport and metabolism (E, H, F, I and P), signal transduction (T), transcription (K), intracellular trafficking (U), RNA processing and modification (A), cell cycle control, cell division, chromosome partitioning (D), chromatin structure and dynamics (B), secondary metabolites biosynthesis, transport and catabolism (Q), and defense mechanisms (V). Most of the identified proteins were assigned as proteins of unknown functions (NK - 15% and 23%).

4. Discussion

Acanthamoeba spp. have two stages in their life cycle, a dormant, free-living cyst stage, with minimal metabolic activity, and the infective trophozoite stage (Siddiqui and Khan, 2012a). Trophozoites of *Acanthamoeba* spp. are infective for a variety of mammalian hosts and can provoke infections in humans, as result of complex interactions between the pathogen-host, environment and even endosimbionts. Much of the damage caused by trophozoites in human corneal or brain infections is the result of several different pathogenic mechanisms not elucidated at the molecular level so far. The elucidation of such mechanisms depends on the identification of proteins involved in the pathogen-host interplay, and, for that, comprehensive proteomic studies are necessary.

Although *A. polyphaga* has not yet a completely sequenced genome, the genetic similarity among *Acanthamoeba* species along with advances in mass spectrometry technologies and protein identification softwares allowed a very efficient identification of *A. polyphaga* proteins. In the present work, we combined complementary experimental strategies for analyzing the *A. polyphaga* trophozoite proteome and presented the largest proteome dataset of *Acanthamoeba* spp. to date. These MS/MS analysis allowed also quantitative estimations of the resolved proteins for future comparative studies (between cyst and trophozoite stages, or between infective and non-infective strains, for example).

Bouyer et al. (2009) performed two-dimensional gel electrophoresis to compare protein expression in trophozoite and cyst forms of *A. castellanii* and five of the identified proteins (translationally-controlled tumor protein, actophorin, elongation factor 2, heat shock protein 70, fructose bisphosphate aldolase and enolase) were common to orthologous proteins identified on the proteome analysis of *A. polyphaga* trophozoite described in this work. Among the protozoa, proteomic analysis of the proteins expressed by cysts of *E. histolytica* were did and a total of 417 non-redundant proteins were identified even when using more sensitive techniques (LC-MS/MS mass spectrometer (Orbitrap) than those used in this study (Ali et al., 2012). While 2-DE-ESI-Q-TOF MS/MS analysis performed in this study, generated a reference map that included 136 identified proteins of 370 spots detected, the reference proteome of *Leishmania* (*Viannia) braziliensis* contain 101 proteins representing 75 protein entries identified by MALDI-TOF-TOF (Cuervo et al., 2007) and the proteome reference maps of *Trichomonas vaginalis* with different pH ranges (pH 3–10, pH 4–7, pH 6–11), had a total of 247 spots representing 164 different proteins (Huang et al., 2009).

Our 2-DE analyses showed evidence of post-translational processing for several *A. polyphaga* trofozoite proteins, in the form of more than one spot assigned to the same identified protein. Post-translational modifications modulate the activity of most eukaryotic proteins and can determine their localization, turnover, and interactions with other proteins (Mann and Jensen, 2003). The detected protein variants or isoforms may be the result of biologically important post-translational modifications, ranging from chemical modifications to proteolytic cleavage (Ambatipudi et al., 2006). Information about post-translational modifications of proteins in *Acanthamoeba* spp. is scarce, but our findings indicate that such phenomena may be frequent. Among the identified proteins with apparent post-translational modifications, we found heat shock protein HSP80, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chaperone DnaK, thioredoxin peroxidase, transaldolase, citrate synthase, enolase, 14-3-3 protein, actin I, beta-tubulin and S-adenosyl-L-homocysteinase. The biological significance of post-translational modification varies according to the type of modifications and particular protein. For example, GAPDH enzyme can be a target of several covalent modifications and it has been implicated in many activities unrelated to glycolysis, such as membrane fusion, binding to host proteins and signal transduction (Sirover, 2005). Alvarez et al. (2007) reported that GAPDH and cysteine synthase A are the main ADP-ribosylated proteins in the *E. histolytica* extracellular medium and may play an important role in ameba survival or in interaction with cells or molecules from the host, as occurs in other organisms.

The GO and KOG analyses carried out to functional annotate the identified resulted in data comparable to those of reference proteomes of others parasites (Huang et al., 2009; Huang et al., 2012; Pawar et al., 2012b). Most of the identified proteins

were assigned to the cellular processes and metabolism classes, represented in the KOG database as energy production and conversion (C); carbohydrate transport and metabolism (G) post-translational modification, protein turnover and chaperones (O), cytoskeleton (Z) and translation, ribosomal structure and biogenesis (J). These results are in agreement with previous microarray and EST analyses, which revealed that genes related to C, G, Z, J and O categories are predominantly overexpressed in trophozoite stage in comparison to the cyst stage (Moon et al., 2011). These results would be expected, because the active trophozoite stage exhibits vegetative growth and increased cellular activity.

Some of the identified metabolism-related proteins in the *A. polyphaga* trophozoite proteome, especially those with the biological functions of energy and carbohydrate metabolism, such as GAPDH , enolase, transaldolase, citrate synthase, pyruvate dehydrogenase and malate dehydrogenase, have been also described as multifunctional. For example, enolase, GAPDH and aldolase from a variety of pathogenic organisms have the ability to bind plasminogen, which may induce plasmin-mediated proteolysis, degrading the extracellular matrix and facilitating invasion and migration within the host (Sotillo et al., 2010; Wang et al., 2011). These proteins may be related the mechanisms of parasite-host interaction and also the evasion of the immune response in infectious process by *A. polyphaga*, becoming interesting targets for future studies.

The cytoskeleton-associated protein group was the second more numerous one. This group contains structural proteins (actin, actin-like protein) and proteins that regulate the stability of the polymers made by these molecules (coronin, actophorin,

profilin, alpha-tubulin, beta-tubulin). Many biological processes, such as cell motility and morphological transformation, require remodeling of cytoskeleton in response to intracellular and extracellular signals. The ability to undergo morphological changes is directly related to virulence and pathogenesis in *Acanthamoeba* spp. Previous studies have shown that morphological transformation occurs when *A. culbertsoni* attaches to collagen and laminin and actin rearrangement was found to be requisite for invasion (Rocha-Azevedo et al., 2009). For such transformation to occur in mammalian cells and parasitic protozoa, the actin cytoskeleton must undergo rearrangement (Berrier and Yamada, 2007; Rios et al., 2008) in order to establish focal points of adhesion (Martin et al., 2002). In addition, data suggest that the interaction between *Acanthamoeba* spp. and extracellular matrix is mediated by protein receptors that can induce major cytoskeletal rearrangements. Such rearrangements have been shown to lead to conformational changes, and may be followed by activation of signal transduction pathways that effect motility and protease secretion (Rocha-Azevedo et al., 2009; Rocha-Azevedo et al., 2010). The identification of these cytoskeleton proteins provided a venue for future studies on the role of cytoskeletal proteins on the pathogenicity of this parasite.

The group of proteins related to post translation modification, protein turnover and chaperones, which includes HSP70, HSP90, proteasome, 14-3-3 protein, chaperone DnaK, peroxiredoxin, ubiquitin, calreticulin, among others, was also well represented in the *A. polyphaga* trophozoite proteome. Several of these proteins are potentially involved in parasite survival mechanisms and may be relevant for *A. polyphaga* pathogenicity. HSP70 was previously identified as antigenic protein by 2DE immunoblot experiments using infected rat serum with *A. polyphaga* (unpublished

results). Heat shock proteins, such as HSP70s, are considered as inducible protective proteins critical for parasite survival and immune-reactive proteins important in parasitic infection (Wang et al., 2009).

Antioxidant proteins play an important role in parasite-mediated anti-cytotoxic and proinflammatory responses against reactive oxygen species generated by the host immune response (Dzik, 2006). Peroxiredoxin is known to play a central role in H_2O_2 detoxification. Thioredoxin peroxidase – peroxiredoxin, identified in this study, suggests that *A. polyphaga* trophozoites release peroxiredoxin, which acts as a protection mechanism against H_2O_2. Peroxiredoxin also was characterized in the proteome of *Naegleria fowleri* and *Toxoplasma gondii* as important antigenic protein, implicated in host cell invasion and facilitated immune response suppression of hosts (Kim et al., 2009; Ma et al., 2009).

Although the *A. polyphaga* trophozoite protein extracts analyzed were enriched in soluble proteins, several membrane proteins were also identified. Membrane proteins are very hard to solubilize by commonly used solubilization buffers generally leading to their underrepresentation in 2-DE (Gorg et al., 2004). ABC transporter and laminin-binding proteins were among the identified membrane proteins. The ATP-binding cassette (ABC) superfamily is one of the largest protein families with representatives in all kingdoms of life. Members of this superfamily are involved in a wide variety of transport processes and recent studies have focused the role of characterized ABC transporters in the biology parasites, including in mechanisms of drug resistance (Sauvage et al., 2009; Leprohon et al., 2011).

Laminin-binding proteins have been described in pathogenic microorganisms, including bacteria (de et al., 2006), fungi (Gonzalez et al., 2005), and protozoa (Petropolis et al., 2008), suggesting that laminin recognition may be a critical step for progression of invasive infection. Laminin-binding proteins in *Acanthamoeba* spp. also have been reported (Kong et al., 2001). Rocha-Azevedo et al. (2009) identified the presence of a 55-kDa laminin-binding protein in pathogenic amoebae *A. culbertsoni*, but it was not found in non-pathogenic amoebae *A. astronyxis*. The identification of these membrane proteins opens possibilities for future studies on the pathogenesis, virulence factors, and drug interaction in *A. polyphaga* and other acanthamoebas.

Our proteomic analysis identified, for the first time the repertoire of proteins expressed by invasive forms of *A. polyphaga*. The present proteomic analysis provided a foundation for the prospective, comparative and functional studies of *A. polyphaga* proteins involved in crucial molecular mechanisms relevant for the development, survival and pathogenicity of this pathogen. The now available *A. polyphaga* proteome map will be a useful reference for future comparative studies associated to the identification of antigenic proteins. Besides, proteomic analyses of the same strain under different culture conditions and of different pathogenic and non-pathogenic strains will allow the identification of differentially expressed or processed proteins. Upon the identification of relevant antigens and differentially expressed proteins, it will be possible to experimentally address the function of these proteins, for the elucidation of mechanisms of pathogen survival and virulence.

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Figures and Tables

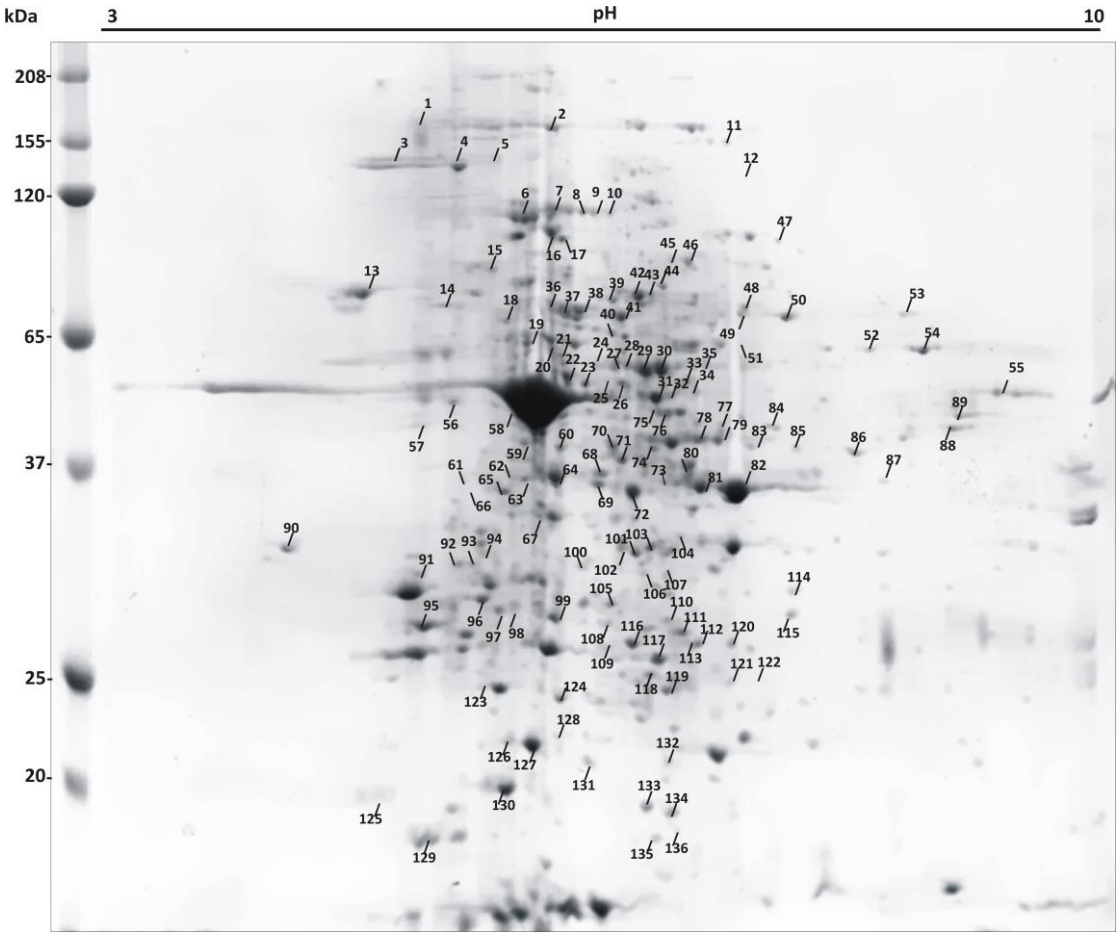

Fig. 1. Representative 2-DE reference map of *A. polyphaga* trophozoite proteins. The proteins were separated on a linear pH range of 3-10 using IEF in the first dimension and 12% SDS-PAGE in the second dimension. Proteins were stained with Coomassie Brilliant Blue G. Molecular mass markers are shown on the left and the acid-to-alkaline gradient is from left to right. Spots containing *A. polyphaga* proteins identified by LC-MS/MS are indicated by numbers that refer to spot numbers listed in Table 1 and Supporting Information Table S1.

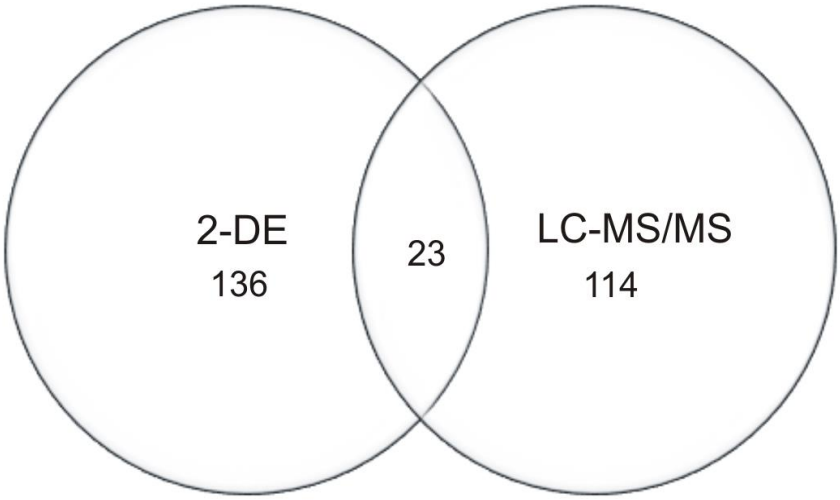

Fig. 2. Venn diagram of the protein sets. Venn diagram illustrating the numbers of protein identifications obtained for each of the two proteome approaches employed in this study.

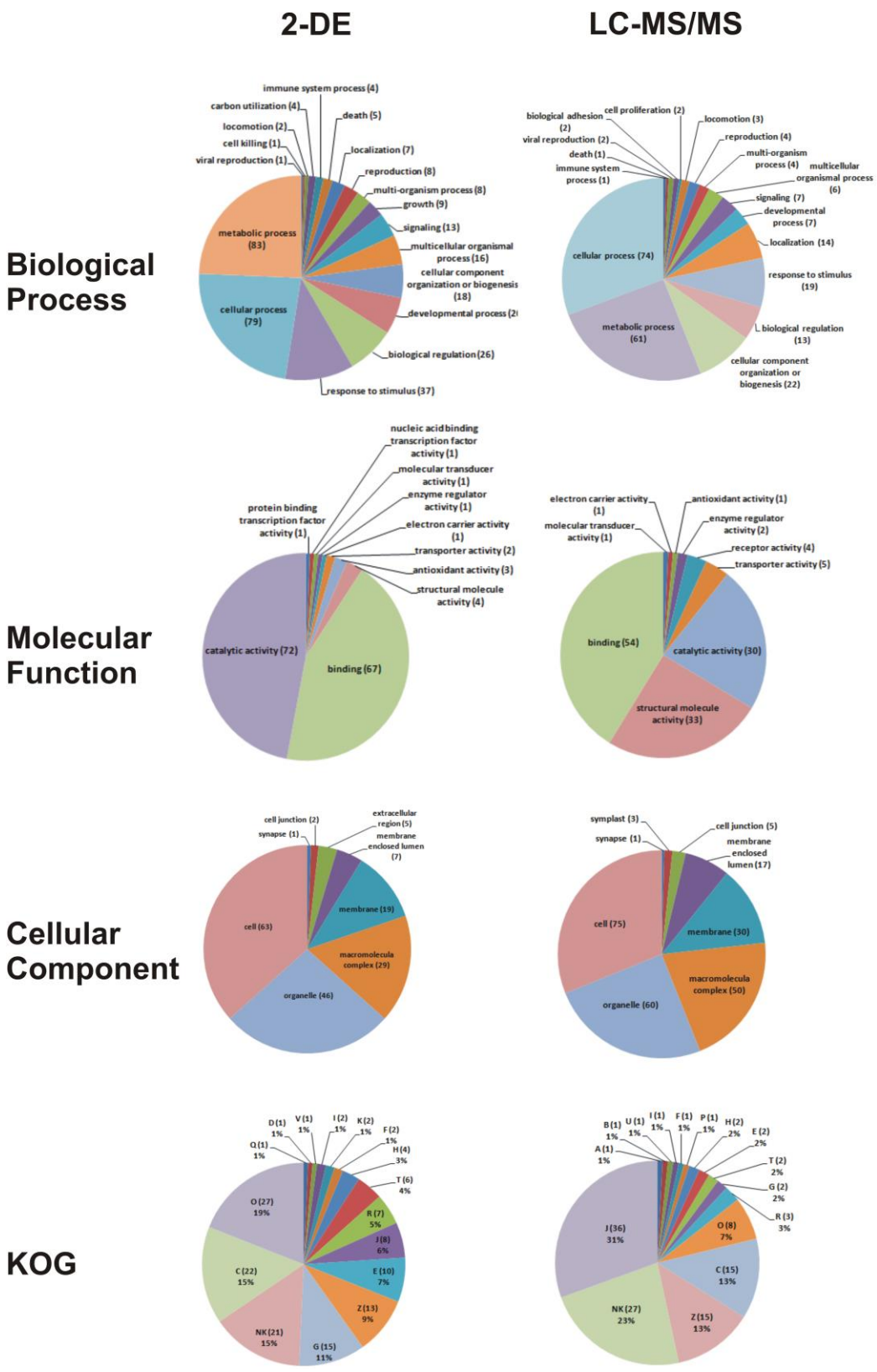
2-DE
LC-MS/MS
Biological Process
immune system process (4)
carbon utilization (4)
death (5)
locomotion (2)
cell killing (1)
localization (7)
viral reproduction (1)
reproduction (8)
multi-organism process (8)
growth (9)
metabolic process (83)
signaling (13)
multicellular organismal process (16)
cellular component organization or biogenesis (18)
cellular process (79)
developmental process (2
biological regulation (26)
response to stimulus (37)
biological adhesion (2)
cell proliferation (2)
locomotion (3)
viral reproduction (2)
reproduction (4)
death (1)
multi-organism process (4)
multicellular organismal process (6)
immune system process (1)
signaling (7)
developmental process (7)
localization (14)
cellular process (74)
response to stimulus (19)
metabolic process (61)
biological regulation (13)
cellular component organization or biogenesis (22)
Molecular Function
nucleic acid binding transcription factor activity (1)
molecular transducer activity (1)
enzyme regulator activity (1)
protein binding transcription factor activity (1)
electron carrier activity (1)
transporter activity (2)
antioxidant activity (3)
structural molecule activity (4)
catalytic activity (72)
binding (67)
electron carrier activity (1)
antioxidant activity (1)
molecular transducer activity (1)
enzyme regulator activity (2)
receptor activity (4)
transporter activity (5)
binding (54)
catalytic activity (30)
structural molecule activity (33)
Cellular Component
cell junction (2)
extracellular region (5)
synapse (1)
membrane enclosed lumen (7)
membrane (19)
cell (63)
macromolecula complex (29)
organelle (46)
symplast (3)
cell junction (5)
synapse (1)
membrane enclosed lumen (17)
cell (75)
membrane (30)
macromolecula complex (50)
organelle (60)
KOG
D (1) 1%
V (1) 1%
I (2) 1%
K (2) 1%
F (2) 1%
Q (1) 1%
H (4) 3%
T (6) 4%
O (27) 19%
R (7) 5%
J (8) 6%
C (22) 15%
E (10) 7%
Z (13) 9%
NK (21) 15%
G (15) 11%
B (1) 1%
U (1) 1%
I (1) 1%
F (1) 1%
P (1) 1%
H (2) 2%
E (2) 2%
A (1) 1%
T (2) 2%
G (2) 2%
R (3) 3%
J (36) 31%
O (8) 7%
C (15) 13%
NK (27) 23%
Z (15) 13%

Fig. 3. Functional analysis of identified proteins from *A. polyphaga* trophozoite. The functional annotation of proteins identified was based on Gene Ontology and Eukaryotic Orthologous Group (KOG) classifications. Proteins were annotated according with biological processes, molecular functions and cellular components terms (level 2) using Blast2GO tool. Distribution of the identified proteins in each category is indicated in the sectors of circle. KOG functional categories are represented in percentages and numbers (in parentheses) of identified proteins in each functional category and indicated in the sectors of circle. KOG functional categories: (C) Energy production and conversion; (O) Posttranslational modification, protein turnover, chaperones; (Z) Cytoskeleton; (G) Carbohydrate transport and metabolism; (J) Translation, ribosomal structure and biogenesis; (E) Amino acid transport and metabolism; (R) General function prediction only; (I) Lipid transport and metabolism; (F) Nucleotide transport and metabolism; (H) coenzyme metabolism; (T) Signal transduction mechanisms; (U) Intracellular trafficking, secretion, and vesicular transport; (A) RNA processing and modification; (J) Translation, ribosomal, structure and biogenesis; (Q) Secondary metabolites biosynthesis, transport and catabolism; (K) Transcription; (D) Cell cycle control, cell division, chromosome partitioning; (V) Defense mechanisms; (P) Inorganic ion transport and metabolism; (B) chromatin structure and dynamics; (NK) protein not related to any KOG category. The number of proteins in the graphic might exceed the total of identified proteins because some were grouped in more than one functional category.

Table 1. Identification of the proteins expressed in *A. polyphaga* trophozoite by 2-DE/ESI-Q-Tof MS/MS

Spot number[a]	Protein name[b]	Accession number	MASCOT score[c]	Sequence coverage (%)	KOG[d]
1	Actin-binding protein (similar to human filamin B, beta)	gi|106788219|gb|EC106596.1|EC106596	40	25	Z
2	Hsp70 chaperone Hsp88	gi|106785681|gb|EC104058.1|EC104058	50	9	O
3	**Heat shock protein - similar to plant Hsp80 and *Dictyostelium* Hsp90**	gi|106787047|gb|EC105424.1|EC105424	33	2	O
4	ER lumenal binding protein - glucose-regulated heat shock 70 protein	gi|106792868|gb|EC111245.1|EC111245	125	16	O
5	**Heat shock protein - similar to plant Hsp80 and *Dictyostelium* Hsp90**	gi|106791400|gb|EC109777.1|EC109777	68	10	O
6	Shock protein 70 (Hsp70)	gi|106790999|gb|EC109376.1|EC109376	368	31	O
7	**Glyceraldehyde-3-phosphate dehydrogenase**	gi|106792440|gb|EC110817.1|EC110817	153	12	G
8	**Chaperone DnaK**	gi|106784777|gb|EC103154.1|EC103154	255	20	O
9	**Chaperone DnaK**	gi|106784777|gb|EC103154.1|EC103154	188	20	O
10	**Chaperone DnaK**	gi|106787484|gb|EC105861.1|EC105861	60	5	O
11	Major vault protein	gi|106792351|gb|EC110728.1|EC110728	56	4	No KOG
12	Mitochondrial aconitase	gi|106789119|gb|EC107496.1|EC107496	33	7	CE
13	Calcium-binding protein – calreticulin	gi|106790960|gb|EC109337.1|EC109337	109	16	O
14	**Beta-tubulin**	gi|106790017|gb|EC108394.1|EC108394	53	6	Z
15	**Beta-tubulin**	gi|106790742|gb|EC109119.1|EC109119	167	17	Z
16	**Vacuolar H+-ATPase subunit A**	gi|106791542|gb|EC109919.1|EC109919	52	10	C
17	**Vacuolar H+-ATPase subunit A**	gi|106791542|gb|EC109919.1|EC109919	92	13	C
18	Alpha-tubulin	gi|106791294|gb|EC109671.1|EC109671	185	28	Z
19	26S proteasome regulatory subunit T3	gi|106784268|gb|EC102645.1|EC102645	33	4	O
20	Translation initiation factor 4A (eIF4a)	gi|106792767|gb|EC111144.1|EC111144	134	17	J
21	4-hydroxyphenylpyruvate dioxygenase	gi|106789239|gb|EC107616.1|EC107616	91	13	E
22	Phosphoglycerate kinase 2-like isoform 2	gi|106780282|gb|EC098659.1|EC098659	82	35	G
23	**NADP-isocitrate dehydrogenase**	gi|106792400|gb|EC110777.1|EC110777	144	16	C
24	Homogentisate 1,2-dioxygenase	gi|106790956|gb|EC109333.1|EC109333	43	5	E

Spot number[a]	Protein name[b]	Accession number	Mascot score[c]	Sequence coverage (%)	KOG[d]
25	**NADP-isocitrate dehydrogenase**	gi|106792400|gb|EC110777.1|EC110777	85	12	C
26	**NADP-isocitrate dehydrogenase**	gi|106789161|gb|EC107538.1|EC107538	120	16	C
27	**Rab GDP dissociation inhibitor**	gi|106789282|gb|EC107659.1|EC107659	87	25	O
28	Glutathione S-transferase domain-containing protein	gi|106785594|gb|EC103971.1|EC103971	64	12	J
29	**Rab GDP dissociation inhibitor**	gi|106789282|gb|EC107659.1|EC107659	171	33	O
30	Hydroxymethylglutaryl-CoA synthase	gi|106781522|gb|EC099899.1|EC099899	107	29	I
31	Methionine adenosyltransferase	gi|106784582|gb|EC102959.1|EC102959	207	25	H
32	S-adenosyl-L-methionine synthetase	gi|106792634|gb|EC111011.1|EC111011	149	15	H
33	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	gi|106790300|gb|EC108677.1|EC108677	69	9	C
34	Mitochondrial elongation factor Tu (EF-Tu)	gi|106792654|gb|EC111031.1|EC111031	170	11	J
35	Actin-like protein	gi|106786253|gb|EC104630.1|EC104630	59	8	Z
36	**Enolase**	gi|106788949|gb|EC107326.1|EC107326	55	7	G
37	**Enolase**	gi|106791007|gb|EC109384.1|EC109384	89	12	G
38	**Enolase**	gi|106792561|gb|EC110938.1|EC110938	176	16	G
39	Vacuolar ATPase B subunit (VatB)	gi|106789110|gb|EC107487.1|EC107487	54	17	C
40	**S-adenosyl-L-homocysteine hydrolase**	gi|106791728|gb|EC110105.1|EC110105	188	17	H
41	**S-adenosyl-L-homocysteinase**	gi|106789297|gb|EC107674.1|EC107674	93	21	H
42	5-phosphoribosyl 5-aminoimidazole-4-carboxamide transformylase (purH)	gi|106791790|gb|EC110167.1|EC110167	185	14	F
43	**Cytosolic aminopeptidase-like protein**	gi|106791503|gb|EC109880.1|EC109880	107	12	R
44	**Cytosolic aminopeptidase-like protein**	gi|106791503|gb|EC109880.1|EC109880	217	26	R
45	Hybrid-cluster protein Hcp (*Entamoeba histolytica*)	gi|106790385|gb|EC108762.1|EC108762	36	4	No KOG
46	Malic enzyme	gi|106785210|gb|EC103587.1|EC103587	83	18	C
47	Cytosolic phosphoenolpyruvate carboxykinase (Pck)	gi|106791934|gb|EC110311.1|EC110311	38	4	C
48	Biotinylated CAP Trapper method (Carninci et al) *A. castellanii* cDNA	gi|106784572|gb|EC102949.1|EC102949	49	4	No KOG
49	Mitochondrial aldehyde dehydrogenase	gi|106788755|gb|EC107132.1|EC107132	37	13	C

Spot number[a]	Protein name[b]	Accession number	Mascot score[c]	Sequence coverage (%)	KOG[d]
50	Succinyl CoA-acetoacetate CoA transferase	gi|106790604|gb|EC108981.1|EC108981	31	4	C
51	G protein pathway suppressor 1 Gps1	gi|106789728|gb|EC108105.1|EC108105	33	5	OT
52	**Citrate synthase**	gi|106786154|gb|EC104531.1|EC104531	74	6	C
53	Adenylyl cyclase-associated protein (CAP)	gi|106791711|gb|EC110088.1|EC110088	153	16	ZT
54	**Citrate synthase**	gi|106786154|gb|EC104531.1|EC104531	98	11	C
55	Malate dehydrogenase	gi|106787209|gb|EC105586.1|EC105586	182	17	C
56	Serine/threonine protein kinase	gi|106786472|gb|EC104849.1|EC104849	206	31	No KOG
57	Translation initiation factor 3, subunit 3 - eIF3-3	gi|106792643|gb|EC111020.1|EC111020	126	12	J
58	**Actin I**	gi|106792826|gb|EC111203.1|EC111203	295	30	Z
59	Adenosine kinase	gi|106792945|gb|EC111322.1|EC111322	41	3	G
60	**CBS-domain-containing protein**	gi|106783924|gb|EC102301.1|EC102301	34	4	C
61	Laminin-binding protein	gi|106782650|gb|EC101027.1|EC101027	49	10	J
62	**Transaldolase**	gi|106790957|gb|EC109334.1|EC109334	76	8	G
63	**Transaldolase**	gi|106792618|gb|EC110995.1|EC110995	165	19	G
64	**Actin I**	gi|106792894|gb|EC111271.1|EC111271	259	26	Z
65	Mitochondrial phosphoenolpyruvate carboxykinase	gi|106788938|gb|EC107315.1|EC107315	33	8	No KOG
66	Pyruvate dehydrogenase E1 beta subunit	gi|106792447|gb|EC110824.1|EC110824	102	11	C
67	Constitutive photomorphogenic (Cop) protein	gi|106789801|gb|EC108178.1|EC108178	101	20	OT
68	Protein kinase	gi|106789862|gb|EC108239.1|EC108239	99	14	C
69	Nucleoside-diphosphate kinase	gi|106781834|gb|EC100211.1|EC100211	196	39	No KOG
70	**Translation elongation factor 2 (EF-2)**	gi|106788630|gb|EC107007.1|EC107007	34	10	J
71	NAD-dependent epimerase/dehydratase	gi|106791424|gb|EC109801.1|EC109801	136	8	No KOG
72	Cytosolic NAD-dependent malate dehydrogenase	gi|106791267|gb|EC109644.1|EC109644	125	16	C
73	TolA-like protein	gi|106786561|gb|EC104938.1|EC104938	44	5	No KOG
74	**Translation elongation factor 2 (EF-2)**	gi|106788630|gb|EC107007.1|EC107007	36	10	J

Spot number[a]	Protein name[b]	Accession number	Mascot score[c]	Sequence coverage (%)	KOG[d]
75	Alcohol dehydrogenase class 3	gi|106789572|gb|EC107949.1|EC107949	117	22	Q
76	**Mitochondrial pyruvate dehydrogenase E1 alpha subunit**	gi|106792789|gb|EC111166.1|EC111166	325	24	C
77	**Mitochondrial pyruvate dehydrogenase E1 alpha subunit**	gi|106792789|gb|EC111166.1|EC111166	124	17	C
78	**Metallo-beta-lactamase family protein**	gi|106786697|gb|EC105074.1|EC105074	177	12	R
79	**Metallo-beta-lactamase family protein**	gi|106786697|gb|EC105074.1|EC105074	205	22	R
80	Fructose-bisphosphate aldolase	gi|106788317|gb|EC106694.1|EC106694	41	12	G
81	**Glyceraldehyde-3-phosphate dehydrogenase**	gi|106792972|gb|EC111349.1|EC111349	265	24	G
82	**Glyceraldehyde-3-phosphate dehydrogenase**	gi|106792972|gb|EC111349.1|EC111349	226	17	G
83	Isocitrate dehydrogenase	gi|106783431|gb|EC101808.1|EC101808	34	11	E
84	**Glyceraldehyde-3-phosphate dehydrogenase**	gi|106792440|gb|EC110817.1|EC110817	147	12	G
85	**Actin-related protein (similar to 2/3 complex, subunit 1)**	gi|106791694|gb|EC110071.1|EC110071	46	7	Z
86	**Actin-related protein (similar to 2/3 complex, subunit 1)**	gi|106791456|gb|EC109833.1|EC109833	219	19	Z
87	**CBS domain-containing protein**	gi|106789845|gb|EC108222.1|EC108222	33	6	E
88	Mitochondrial NAD+-specific isocitrate dehydrogenase gamma subunit	gi|106791881|gb|EC110258.1|EC110258	140	5	E
89	Aspartate aminotransferase	gi|106782991|gb|EC101368.1|EC101368	42	5	E
90	ABC transporter permease	gi|106787787|gb|EC106164.1|EC106164	45	7	No KOG
91	**14-3-3 family protein ArtA, putative**	gi|106780833|gb|EC099210.1|EC099210	78	22	O
92	**14-3-3-like regulatory protein**	gi|106791130|gb|EC109507.1|EC109507	77	9	O
93	**14-3-3-like regulatory protein**	gi|106792166|gb|EC110543.1|EC110543	99	12	O
94	**14-3-3-like regulatory protein**	gi|106791130|gb|EC109507.1|EC109507	53	9	O
95	**Nascent polypeptide-associated complex subunit alpha**	gi|106789431|gb|EC107808.1|EC107808	84	21	K
96	Inorganic diphosphatase	gi|106783526|gb|EC101903.1|EC101903	151	12	C
97	**Subunit of heterodimeric actin capping protein cap32/34**	gi|106787421|gb|EC105798.1|EC105798	256	21	Z
98	**Subunit of heterodimeric actin capping protein cap32/34**	gi|106787421|gb|EC105798.1|EC105798	99	14	Z
99	Phytanoyl-CoA dioxygenase	gi|106781904|gb|EC100281.1|EC100281	94	16	No KOG

Spot number[a]	Protein name[b]	Accession number	Mascot score[c]	Sequence coverage (%)	KOG[d]
100	3-hydroxyanthranilate 3,4-dioxygenase	gi|106791370|gb|EC109747.1|EC109747	85	20	E
101	**Protein phosphatase 2C**	gi|106790603|gb|EC108980.1|EC108980	249	36	T
102	**Protein phosphatase 2C**	gi|106790603|gb|EC108980.1|EC108980	249	36	T
103	**CBS domain-containing protein**	gi|106791495|gb|EC109872.1|EC109872	135	11	C
104	Phosphoribosylpyrophosphate synthetase	gi|106790317|gb|EC108694.1|EC108694	61	19	FE
105	Actin-capping protein alpha chain	gi|106792342|gb|EC110719.1|EC110719	256	20	Z
106	Proteasome subunit alpha type - prosome - macropain (Psma1)	gi|106789770|gb|EC108147.1|EC108147	72	20	O
107	**CBS domain-containing protein**	gi|106779788|gb|EC098165.1|EC098165	109	57	No KOG
108	Methyltransferase domain protein	gi|106787197|gb|EC105574.1|EC105574	156	15	R
109	**Triosephosphate isomerase**	gi|106789176|gb|EC107553.1|EC107553	68	16	G
110	Proteasome subunit - similar to human alpha type 6 (Psma6)	gi|106790861|gb|EC109238.1|EC109238	141	18	O
111	Hypothetical protein	gi|106780583|gb|EC098960.1|EC098960	40	12	No KOG
112	Short-chain dehydrogenase/reductase family protein	gi|106785853|gb|EC104230.1|EC104230	216	24	R
113	Proteasome (prosome, macropain) subunit (similar to beta type 7)	gi|106790837|gb|EC109214.1|EC109214	60	11	O
114	**Phosphoenolpyruvate mutase**	gi|106792620|gb|EC110997.1|EC110997	396	30	No KOG
115	**Phosphoenolpyruvate mutase**	gi|106792620|gb|EC110997.1|EC110997	223	24	No KOG
116	**Triosephosphate isomerase**	gi|106789176|gb|EC107553.1|EC107553	150	36	G
117	**Phosphoglycerate mutase**	gi|106789665|gb|EC108042.1|EC108042	118	16	G
118	**LIM-type zinc finger-containing protein**	gi|106782384|gb|EC100761.1|EC100761	89	13	No KOG
119	Quinoid dihydropteridine reductase	gi|106791445|gb|EC109822.1|EC109822	114	10	E
120	Short-chain enoyl-CoA hydratase	gi|106787552|gb|EC105929.1|EC105929	187	25	I
121	**LIM-type zinc finger-containing protein**	gi|106782384|gb|EC100761.1|EC100761	156	20	No KOG
122	Proteasome (prosome, macropain) subunit (similar to beta type 1)	gi|106791102|gb|EC109479.1|EC109479	37	5	O
123	**Nascent polypeptide-associated complex alpha polypeptide (Naca)**	gi|106789431|gb|EC107808.1|EC107808	84	21	K
124	Conserved hypothetical protein	gi|106782193|gb|EC100570.1|EC100570	68	22	No KOG

Spot number[a]	Protein name[b]	Accession number	Mascot score[c]	Sequence coverage (%)	KOG[d]
125	Cytosolic glycoprotein FP21	gi|4528553|gb|AT001460.1|AT001460	76	25	O
126	**Thioredoxin peroxidase – peroxiredoxin**	gi|106789626|gb|EC108003.1|EC108003	237	31	O
127	**Thioredoxin peroxidase – peroxiredoxin**	gi|106789892|gb|EC108269.1|EC108269	423	38	O
128	Rho GDP-dissociation inhibitor	gi|106790160|gb|EC108537.1|EC108537	192	25	T
129	Human tumor protein, translationally-controlled	gi|106788929|gb|EC107306.1|EC107306	141	37	DZ
130	Eukaryotic translation initiation factor 5A	gi|106780760|gb|EC099137.1|EC099137	49	15	J
131	**Thioredoxin peroxidase – peroxiredoxin**	gi|106789369|gb|EC107746.1|EC107746	102	18	O
132	Proteasome subunit	gi|106790486|gb|EC108863.1|EC108863	133	15	O
133	UspA domain-containing protein	gi|106785762|gb|EC104139.1|EC104139	50	4	No KOG
134	Lysozyme (N-acetylmuraminidase)	gi|106788669|gb|EC107046.1|EC107046	53	13	No KOG
135	PfpI family peptidase	gi|106790308|gb|EC108685.1|EC108685	59	10	RV
136	gfo/Idh/MocA family oxidoreductase	gi|106779176|gb|EC097553.1|EC097553	68	45	No KOG

[a]Spots are from 2-DE gels. Spot numbers correspond to those indicated in Fig. 1

[b]Protein with possible post-translational modified are indicated in bold

[c]Mascot score is – 10 x log(P), where P is the probability that the observed match is a random event. Peptide scores > 30 for ESTs database of *Acanthamoeba* spp., indicate identity or extensive homology ($p < 0.05$)

[d]KOG functional categories: (C) Energy production and conversion; (O) Posttranslational modification, protein turnover, chaperones; (Z) Cytoskeleton; (G) Carbohydrate transport and metabolism; (J) Translation, ribosomal structure and biogenesis; (E) Amino acid transport and metabolism; (R) General function prediction only; (I) Lipid transport and metabolism; (F) Nucleotide transport and metabolism; (H) coenzyme metabolism; (T) Signal transduction mechanisms; (Q) Secondary metabolites biosynthesis, transport and catabolism; (K) Transcription; (D) Cell cycle control, cell division, chromosome partitioning; (V) Defense mechanisms and (NK) protein not related to any KOG category.

Supporting Information Available

Supplementary Table A1: Trophozoite proteins identified by 2-DE/ESI-Q-Tof MS/MS.

Supplementary Table A2: Proteins identified in *A. polyphaga* trophozoite by LC-MS/MS.

Supplementary Data B1. Annotated spectra for all single hits

CAPÍTULO III

Manuscrito em preparação para submissão em periódico científico.

Comparative proteomic analysis of *Acanthamoeba polyphaga* attenuated isolate with enhanced virulence after experimental infection of rat and identification of antigenic proteins

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1. Introduction

Acanthamoebae are protozoans living freely in soil and water and have increased in medical importance during the last decade, due to their potential as opportunistic pathogens of infect human hosts. Infection with *Acanthamoeba* spp. can cause severe diseases such as vision-threatening *Acanthamoeba* keratitis, pneumonitis and fatal granulomatous amoebic encephalitis (Visvesvara, 2010; Knickelbein & Chu, 2013). These infections have been recognized as important health problems due to an increase in the number of people wearing contact lenses and a rise in the number of immunocompromised patients (Marciano-Cabral & Cabral, 2003; Visvesvara & Schuster, 2007, Carvalho et al., 2009).

Acanthamoeba spp. have two stages in their life cycle, a dormant, free-living cyst stage, with minimal metabolic activity, and the infective trophozoite stage (Siddiqui & Khan, 2012). Trophozoites of *Acanthamoeba* spp. are infective for a variety of mammalian hosts, as result of complex interactions between the pathogen-host and environment. Much of the damage caused by trophozoites in human corneal or brain infections is the result of several different pathogenic mechanisms not elucidated at the molecular level so far. Previous studies have identified a number of *Acanthamoeba* spp. molecules that appear to be linked to virulence, including cysteine proteinases, amoebapores, lectin (manose) and superoxide dismutase (Kim et al., 2012, Tripathi, Smith, Abdi & Alizadeh, 2012; Yoo & Jung, 2012, Michalek et al., 2013). However, the mechanisms of *Acanthamoeba* spp. pathogenicity suggest the existence of several other classes of unidentified virulence factors, such as genes/proteins involved in secretion and/or trafficking of molecules between host and pathogen cells, antioxidant defenses,

protection against cytolytic effects, or evasion and/or modulation of the host immune system.

The classical proteomic approach, two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS) is a powerful tool to capture dynamics of global proteomic changes by simultaneous resolution of large number of cellular proteins. The methodology is particularly advantageous for identification of stress induced proteins along with their post-translational modifications and to correlate altered protein abundance/modifications with physiological function(s) (Beranova-Giorgianni, 2003; Brewis & Brennan, 2010).

Comparative proteomic approaches have been successfully used for the identification virulence factors of many pathogens (Davis et al., 2006; Biller et al., 2009; Hong et al., 2011; Regidor-Cerrillo et al., 2012). These analyzes have revealed a diversity of proteins expressed by different parasitic species, helping to elucidate the molecular mechanisms used for interaction with host species and to identify potential biomarkers for diagnosis and targets for the development of new drugs or vaccines. For *Acanthamoeba* spp., proteomic studies performed thus far were mainly prospective. Results included the 2DE mapping of the *A. polyphaga* low virulent strain ATCC 30872 (Caumo et al., 2013) as well as evidence of post-translational modifications of several *A. polyphaga* proteins. More comprehensive and comparative proteomic approaches are expected to provide an overview of the *A. polyphaga* repertoire of virulence-related proteins.

Amebic virulence is attenuated by the long-term cultivation of amoebae *in vitro* and can be recovered by serial mouse passages via intranasal infection (Xuan et al., 2009). This virulence restoration is likely to be associated with upregulation of

virulence-related genes, whose products may play roles in the pathogenesis of *Acanthamoeba* spp. Therefore, to identify possible virulence factors of *A. polyphaga*, we started investigating changes in the proteome of a long-term *in vitro* cultivated low virulent environmental strain before and after experimental infection. The *A. polyphaga* strain ATCC 30872 proteome was assessed by two-dimensional gel electrophoresis (2DE) followed by identification of protein spots by in tandem mass spectrometry (MS/MS). The 2DE profiles prior and after a passage in rat experimental hosts were compared. Several differentially expressed proteins were identified, shedding light on amoeba mechanisms of pathogenicity.

2. Material and methods

2.1 Animals

Male Wistar rats weighing approximately 200 g were used for the present study. The animals were fed with a standard laboratory diet, kept at 22 ± 2 ºC, 50%-70% humidity, and a 12-hour day-night cycle; drinking water was available ad libitum during the experiments. Experimental procedures involved in the study were in agreement with Brazilian laws and approved by the Animal Ethics Committee of the Federal University of Rio Grande do Sul (process number 18847). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

2.2 *A. polyphaga* strains and cultivation, and cell protein extracts

The *A. polyphaga* strain (ATCC 30872), obtained from the American Type Culture Collection. It is a freshwater environmental isolate regarded as non-pathogenic (Rocha-Azevedo, 2007). Trophozoites were cultured axenically in peptone-yeast-

glucose (PYG) medium, as described previously (Schuster, 2002). Experimental infections in Wistar rats were did intranasally, with $1x10^6$ trophozoites/animal as previously described (Gianinazzi et al., 2009). Rats were checked daily for clinical signs indicating possible central nervous system (CNS) infection, and were euthanized after 25 days of infection, when the clinical status was seriously deteriorated (characterized by weight loss, obtundation, and ataxy). Tissue samples from brain and lungs were collected and used for isolation of viable trophozoites by culture on agar plates (Gianinazzi et al., 2009). Isolated trophozoites were then transferred to PYG medium for axenization.

Protein extracts were obtained from *A. polyphaga* long-term (over 4 years) *in vitro* cultivated trophozoites (*A. polyphaga* prior infection – ApPI – samples), and from *A. polyphaga* trophozoites collected from Wistar rat infected lungs, and *in vitro* cultured axenically in PYG medium (3^{rd} subculture after isolation) (*A. polyphaga* after infection – ApAI – samples). For protein extraction, ApPI and ApAI trophozoites from approximately $1x10^8$ cells (trophozoites at log growth phase) from three identical and independent cultures (biological replicates) were used. Cells were harvested at 2000 x *g* for 10 min and washed twice in phosphate-buffered saline (PBS) buffer (pH 7.2), prior to resuspension in 1 ml of 25 mM Tris–HCl, pH 7.2, containing a cocktail of protease inhibitors. Cell suspensions were then lysed by sonication (25 Hz in a VC601 Sonics and Materials Inc. sonicator) in an ice bath by five 30 s cycles with 1 min interval between pulses. Lysates were centrifuged (18,000 x *g*, 15 min, 4 °C) to separate soluble and insoluble protein fractions. Soluble proteins were quantified using a Qubit™ quantitation fluorometer and Quant-it™ reagents (Invitrogen, USA).

2.3 Two-dimensional gel electrophoresis and gel image analysis

Protein samples (2 mg) were precipitated overnight at -20 °C with two volumes of ice cold 20% (w/v) trichloroacetic acid/acetone. Protein precipitates were recovered by centrifugation (10 min at 18,000 x *g*) and washed five times with ice cold acetone. The pellet was air-dried and solubilized in 350 µL isoelectric focusing (IEF) buffer containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT), and 0.2% (v/v) ampholytes pH 3-10 (Bio-Rad, Hercules, US). The 17 cm immobilised pH gradient (IPG) strips (pH 3-10 or 4-7, Bio-Rad) were passively rehydrated with the cell extract sample in IEF buffer for 16 h and IEF was performed in a Protean IEF cell system (Bio-Rad, Hercules, US) with up to 50,000 VH at a maximum voltage of 10,000 V. Strips were equilibrated for 15 min in equilibration buffer I (30%, v/v, glycerol, 6 M urea, 1% DTT, a trace of bromophenol blue) and for 15 min in equilibration buffer II (equilibration solution I with DTT replaced by 4% iodoacetamide). In the second dimension, IPG strips were run vertically onto SDS-PAGE 12% gels using PROTEAN® II xi 2D Cell (Bio-Rad, Hercules, US). For each protein sample, three independent gels were run (technical replicates). Gels were stained with 0.1% Coomassie Brilliant Blue G (Acros, Geel, Belgium), scanned with a computer-assisted G-800 densitometer (Bio-Rad, Hercules, US). Spot detection, measurement, background subtraction and matching were performed using PDQuest 2-D Gel Analysis Software version 8.0.1 (Bio-Rad, Hercules, CA). Selected spots from individual gel images were first matched to at least two other replicate gels. We considered differentially expressed proteins between two experimental conditions (ApPI and ApAI) as the ones having $p < 0.01$ according to the Student's t test. Fold change 1.5 was determined per group. After automatic spot detection, manual editing was performed on all spots to avoid incorrect match detected by the software. To determine experimental p*I* and Mw coordinates for

each single spot, 2-DE gels were calibrated using a select set of reliable identification landmarks distributed throughout the entire gel. Experiments were performed with three biological and technical replicates.

2.4 Sample preparation for mass spectrometry

Protein spots were manually excised from Coomassie stained 2-DE gels and in-gel digested with trypsin. Gel plugs were treated in three washing steps with 180 µl of 50% acetonitrile and 50 mM ammonium bicarbonate for 15 min, followed by one washing step with 180 µl of acetonitrile. After the washing procedures, gel plugs were dried by vacuum centrifugation and digested for 18-24 h at 37 °C using 12 µl of 10 mg ml^{-1} modified porcine trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega), diluted to 25 mM in NH_4HCO_3. After tryptic digestion, peptides were extracted in two washing steps with 50 ml of 50% acetonitrile and trifluoroacetic acid (TFA) for 1 h. Extracted peptides were dried and resuspended in 10 µl of 0.1% TFA.

2.5 Mass spectrometry analyses

Peptides from digested protein spots were analyzed by on-line liquid chromatography/mass spectrometry (LC-MS/MS) using a Waters nanoACQUITY UPLC system coupled to a Waters Micromass Q-TOF Micro or Q-TOF Ultima API mass spectrometer (Waters MS Technologies, UK). The peptides were eluted from the reverse-phase column toward the mass spectrometer at a flow rate of 200 nl/min with a 10-50% water/ACN 0.1% formic acid linear gradient over 10 min. The MS survey scan was set to 1 s (0.1 s interscan delay) and recorded from 200 to 2000 *m/z*. MS/MS scans were acquired from 50 to 2000 *m/z*, and scan and interscan rates were set as for MS. The samples were run in DDA mode where each full MS scan was followed by three

consecutive MS/MS scans. For each survey scan, the three most intense multiple charged ions over a threshold of 8 counts were selected for MS/MS analysis. The collision energies for peptide fragmentation were set using the charge state recognition files for +2, +3, and +4 peptide ions provided by MassLynx (Waters). MS/MS raw data were processed using ProteinLynx Global Server 2.0 software (Waters), and peak lists were exported in the micromass (.pkl) format. For each protein sample, at least two independent LC-MS/MS runs were performed.

2.6 Database searching and bioinformatics analyses

For peptide identification, all MS/MS data were searched against databases using MASCOT software 2.0 (http://www.matrixscience.com, Matrix Science) with the following parameters: a maximum of one missed cleavage site, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine and a 0.1 mass unit tolerance on parent and fragment ions. The significance threshold was set at $p < 0.05$, and only peptides with individual ion scores above this significance threshold were considered for protein identification. The MS/MS spectra of protein identifications based on a single peptide and on borderline scores were manually inspected for acceptance.

A local *Acanthamoeba* spp. database was constructed based on the *A. castellanii* and *A. healyi* EST sequences available at GenBank dbEST (http://www.ncbi.nlm.nih.gov/dbEST/) (Jan/2011); ii) 13,770 trophozoites EST sequences of *A. castellanii* available at TBestDB (http://tbestdb.bcm.umontreal.ca) expressed of in normal conditions or genes silenced (O'Brien et al., 2007); and iii) 3,897 ESTs from *Acanthamoeba* EST DB database (http://www.amoeba.or.kr), expressed by amebae under long term *in vitro* culture, mouse brain passage or encystation derived (Moon et al., 2009).

Eukaryotic Orthologous Group (KOG) annotations [23] were assigned based on sequence similarity searches against the KOG annotated proteins (http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html).

2.7 Anti-*Acanthamoeba polyphaga* sera

Four six-week-old Wistar rats were immunosuppressed with dexamethasone (5 mg/kg) during three days and intranasally infected with 1 x 10^6 trophozoites/animal, under anesthesia. The rats were daily checked for clinical signs indicating a possible CNS infection such as loss of weight, obtundation, and ataxy. Antigenic sera generated from each strain were collected from blood. Blood was collected in days 7, 14, 21, 35, 50, 70 after infection. Preimmune sera were used as negative controls. Serum samples were evaluated by ELISA. Specific titles from 1:100 to 1:4000 were obtained and the serum with the highest title was used in the presented immunoblot experiments. When titles of sera started to decrease, the animals were euthanized and tissue samples from brain, lungs, muzzle, and blood were collected and used for isolation of viable amoebae.

2.8 Immunoblotting

Proteins were resolved by 2-DE and electroblotted onto PVDF membranes (GE Healthcare, Chalfont St. Giles, UK) at 20 V for 16–18 h. Membranes were blocked for 1 h with 5% nonfat dry milk in PBS-T (PBS containing 0.1% v/v Tween-20) and then incubated with a pool of four sera from Wistar rats infected at a 1:1000 dilution for 1 h and 30min. After three washes with PBS-T, blots were incubated with a secondary antibody (anti-rat IgG peroxidase-conjugated, Sigma-Aldrich, St.Louis, US) at a 1:5000 dilution for 1 h. The 2-DE blots were revealed with ECL detection reagent (GE

Healthcare) and imaged using the VersaDoc imaging system (Bio-Rad). A pool of four sera from Wistar rats non-infected was used as negative control.

The 2-DE gel and blot images were analyzed using PDQuest 8.0 software (Bio-Rad) for spot detection and matching. Antigenic spots of 2-DE blots were identified based on matches with a 2DE proteomic map. For each protein sample, three independent immunoblot experiments were performed (technical replicates).

3. Results

3.1 Proteins differentially expressed in *A. polyphaga* trophozoites ApPI and ApAI samples

For a comparative investigation of the repertoires of proteins expressed by *A. polyphaga* ATCC 30872 ApPI and ApAI samples, the respective trophozoite protein extracts were analyzed by 2DE in the pH range of 3-10 (Fig. 1). Three independent experiments generated three biological replicates that were analyzed with three technical replicates using the PDQuest 2-D Gel Analysis Software Version 8.0.1 (Bio-Rad). The 2DE protein spot profiles were highly reproducible (~90% matching between replicates), in terms of both the total number of protein spots and their relative positions and intensities. The analysis of each condition revealed an average of 370 spots for the ApPI sample, as previously established proteomic map of *A. polyphaga* (Caumo *et al*., 2013) and for the ApAI sample, a total of 413 spots were resolved.

The semiquantitative analysis of protein expression of *A. polyphaga* trophozoites ApPI and ApAI was performed and Student's t test statistical analysis revealed a total of 51 differentially expressed proteins ($p < 0.05$, spots shown in Fig. 1A and B). Of these, 19 and 32 proteins were upregulated in ApPI and ApAI, respectively. Significant

qualitative (presence versus absence) were identified including 17 spots of ApPI and 58 spots of ApAI. Differences in protein spot intensity, represented as fold change, ranged from 1.5 to 10.4. Figure 1 show representative 2D gels of the two analyzed conditions and indicate spot number of the upregulated in ApAI proteins.

3.2 Identification of differentially expressed proteins

All protein spots of ApAI that showed differential changes in expression of 1.5-fold or greater ($p < 0.05$) were excised, trypsin digested, and identified by ESI-Q-Tof MS/MS analysis, following by proteins database sequence matching. Table 1 corresponds to labels in Figs. 1A and 1B and lists the protein names, *Acanthamoeba* spp. EST database accession numbers, Mascot score, sequence coverage, protein molecular weights observed, isoelectric points (pI) observed, fold change and functional categorization.

Among differentially expressed proteins upregulated in ApAI, isoforms of actin, coronin and peroxidase were identified in both ApAI and ApPI. In addition, ApAI also showed overexpression of peptidase, enolase, peroxiredoxin, CBS, LIM, sinapsin, translation elongation factor Tu (EF-Tu), Heat shock protein 90 alpha, hypothetical proteins and others proteins were identified (Table 1). Significant qualitative proteins in ApAI included 16 proteins identified, among them serine proteinase, serine hydroxymethyltransferase, cysteine proteinase CP1, Rho GDP-dissociation inhibitor, S-adenosyl-L-homocysteinase hydrolase, methylthioadenosine phosphorylase, transmembrane protein, Histone, GAMMA CA3, Putative stress-related protein, Transaldolase, Vacuolar ATPase B subunit (VatB) and several proteins involved in carbohydrate metabolism.

3.3 Identification of antigenic proteins

In order to identify antigenic proteins from *A. polyphaga*, immunoblot assays of 2-DE using the serum of infected rats were performed (Fig. 2). The comparison of the immunoblots with the *A. polyphaga* proteomic map (ApAI) allowed the unambiguous identification of antigenic proteins, namely HSP70, chaperonin GroL, hydroxymethylglutarylCoA synthase, GDP dissociation inhibitor, mitochondrial aspartate aminotransferase, RhoGEF domain containing protein, phosphomannomutase, S-adenosyl-L-homocysteine hydrolase, GAMMA CA3 (GAMMA CARBONIC ANHYDRASE 3) e ubiquinol cytochrome reductase transmembrane region.

3.4 KOG functional classification

According to the KOG functional classification (Fig. 3), nine groups of the proteins upregulated in ApAI were related to (Z) Cytoskeleton, (C) Energy production and conversion, (O) Posttranslational modification, protein turnover, chaperones, (G) Carbohydrate transport and metabolism, (J) Translation, ribosomal structure and biogenesis, (E) Amino acid transport and metabolism, (R) General function prediction only, (F) Nucleotide transport and metabolism and coenzyme metabolism (H). Most of the identified proteins in the ApAI were assigned as proteins of unknown functions (NK), which includes proteins possibly involved in stress putative, lipid-binding protein (membrane proteins) and several hypothetical.

4. Discussion

Our preliminary results indicated that the analyzed environmental and low virulent *A. polyphaga* ATCC 30872 isolate undergoes changes in its expressed protein repertoire upon a single passage in rat hosts, during which it acquires competence to cause disease. These alterations involve several biological processes, since upregulated

proteins include some involved in stress response, proteolysis, energetic metabolism, phosphorylation, cell cycle control and proliferation.

Among the proteins upregulated identified in ApAI, we found the majority classified in groups Z, O, C e R, such as oxidative stress-response molecules (peroxiredoxin) and proteases that play important roles in microbial pathogenesis including host cell and tissue invasion, migration, catabolism of host proteins, and cytoadherence (cysteine proteinase, peptidase M20, 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, serine proteinase).

Antioxidant proteins play an important role in parasite-mediated anti-cytotoxic and proinflammatory responses against reactive oxygen species generated by the host immune response (Dzik, 2006). Peroxiredoxin is known to play a central role in H_2O_2 detoxification. Thioredoxin peroxidase – peroxiredoxin, identified in this study, suggests that *A. polyphaga* trophozoites release peroxiredoxin which acts as a protection mechanism against H_2O_2. Peroxiredoxin also was characterized in the proteome of *Naegleria fowleri* and *Toxoplasma gondii* as important antigenic protein, implicated in host cell invasion and facilitated immune response suppression of hosts (Kim et al., 2009; Ma et al., 2009).

The group of proteins related to post translation modification, protein turnover and chaperones, which includes HSP70, chaperonin GroL among others, were identified as antigenic protein by 2DE immunoblot experiments using infected rat serum with *A. polyphaga*. Heat shock proteins, such as HSP70s, are considered as inducible protective proteins critical for parasite survival and immune-reactive proteins important in parasitic infection (Wang et al., 2009).

Further investigation will be necessary for the confirmation of the differential expression of identified proteins and for their functional characterization. The comparative proteomic analysis performed so far improved our knowledge on the protein repertoire expressed by the trophozoite stage of *A. polyphaga*, and identified proteins up or downregulated of a low virulent strain in the process of its adaptation to a host environment. Functional characterization of some of these differentially expressed proteins will help in the elucidation of at least some of the *A. polyphaga* physiological aspects associated to pathogenicity.

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The authors have declared no conflict of interest.

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Figures and Tables

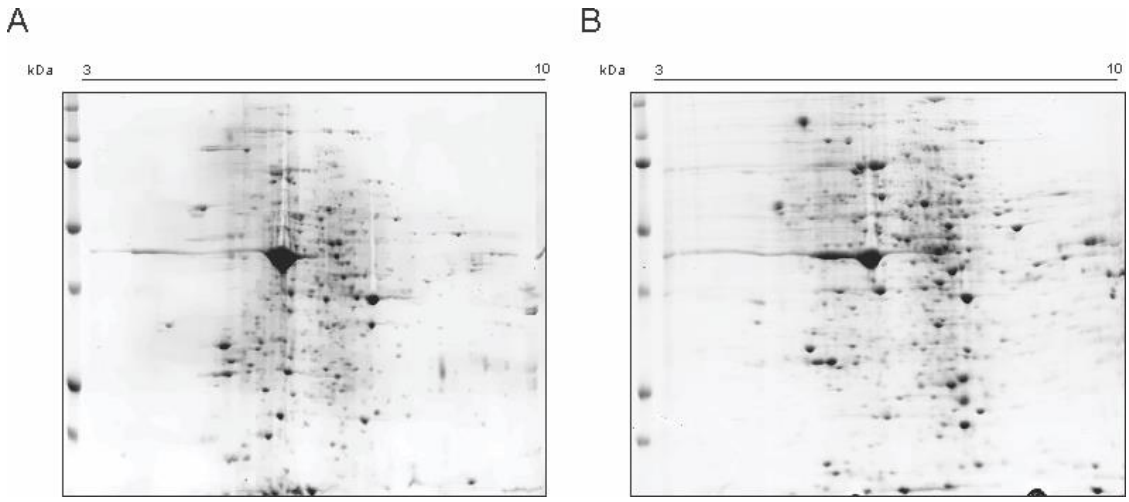

Fig. 1. **2-DE proteome profiling the two *A. polyphaga* samples with IEF at pH 3-10.** Protein samples (2 mg) from the *A. polyphaga* trophozoites ApPI (A) and ApAI (B) were separated by IEF using 17 cm pH 3-10 IPG strips, followed by SDS-PAGE on 12% gels and stained with Coomassie Brilliant Blue G.

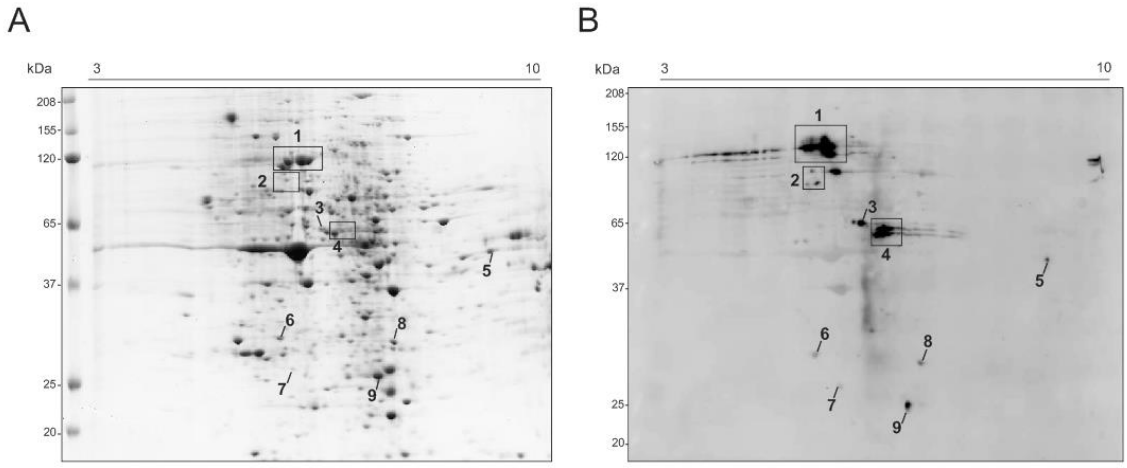

Fig. 2. 2-DE immunoblotting analysis of trophozoites *A. polyphaga* (ApAI) using the serum of infected rats.

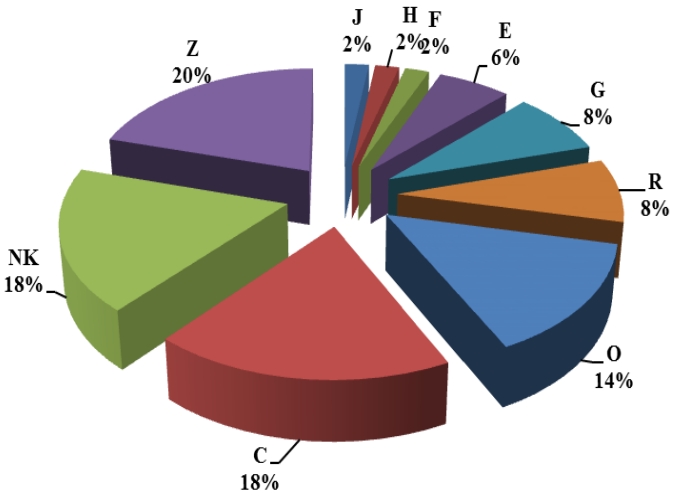

Fig. 3. **Functional analysis of upregulated proteins identified from (ApAI) *A. polyphaga* trophozoites.** The functional annotation of proteins identified was based on Gene Ontology and Eukaryotic Orthologous Group (KOG) classifications. KOG functional categories are represented in percentages of identified proteins in each functional category and indicated in the sectors of circle. KOG functional categories: ((Z) Cytoskeleton; (E) Amino acid transport and metabolism; (O) Posttranslational modification, protein turnover, chaperones; (G) Carbohydrate transport and metabolism; (R) General function prediction only; C) Energy production and conversion; (J) Translation, ribosomal structure and biogenesis; (H) coenzyme metabolism; (F) Nucleotide transport and metabolism; (NK) protein not related to any KOG category. The number of proteins in the graphic might exceed the total of identified proteins because some were grouped in more than one functional category.

Table 1. Upregulated proteins of *A. polyphaga* trophozoites collected from Wistar rat lungs after experimental infection (ApAI) in comparison to low virulent isolate cultivated by long-term in vitro (ApPI). Proteins listed in this table were found to be statistically differentially expressed ($p < 0.05$) and have an absolute fold change greater than 1.5.

Spot Number[a]	**Protein name**	**Accession number (EST)**	**MASCOT score**[b]	**Sequence coverage (%)**	**M_w (kDa) (obs)**	**p*I* (obs)**	**Fold change**	**Molecular function**[c]
1	Factin-capping protein subunit beta	EC105798	268	27	28.16	5.19	3.0	Z
2	Peptidase M20-like protein	EC108585	28	5	59.03	5.28	3.12	E
3	Peroxiredoxin 2	EC108819	255	29	22.80	5.54	10.06	O
4	Enolase	EC110938	515	40	60.65	5.44	3.94	G
5	CBS domain containing protein	EC109872	135	11	32.77	5.92	4.30	O
6	LIM domain containing protein	EC100761	156	20	26.07	6.10	2.02	No KOG
7	Synapsin	EC110457	40	5	61.72	6.08	6.73	No KOG
8	Peroxidase	EC101256	44	7	130.89	5.98	3.51	R
9	Gelation fator	EC106596	187	25	114.19	4.94	2.42	Z
10	Heat shock protein 90 alpha	EC110974	293	44	99.66	5.21	3.92	O
11	Conserved hypothetical protein [Trypanosoma vivax Y486]	gi|106785662	204	21	24.56	5.63	2.50	No KOG
12	Actin-1	EC111336	631	53	37.19	5.44	2.21	Z
13	GDP associated inhibitor	EC109305	309	45	49.25	5.68	2.57	O
14	Phosphogluconate dehydrogenase	EC108664	81	13	56.26	5.68	1.94	G
15	Coronin (actin-binding protein)	EC110773	360	40	61.09	5.79	1.82	Z
16	Transketolase	EC109830	88	9	89.79	5.69	3.39	G
17	Coronin (actin-binding protein)	EC110773	106	11	62.47	5.89	2.46	Z
18	ATP synthase	EC101108	58	7	79.34	5.91	2.59	No KOG
19	Hypothetical protein	EC104642	93	6	20.90	6.14	1.80	No KOG

Spot Number[a]	Protein name	Accession number (EST)	MASCOT score[b]	Sequence coverage (%)	M_w (kDa) (obs)	p*I* (obs)	Fold change	Molecular function[c]
20	Beta1 proteasome1D	EC106755	48	18	22.08	5.96	2.44	O
21	Mitochondrial pyruvate dehydrogenase E1 alpha subunit	EC111166	271	29	40.88	6.13	1.82	C
22	Actin related protein 3	EC104630	279	30	49.46	6.07	2.60	Z
23	Translation elongation factor Tu (EF-Tu)	EC111031	301	42	46.07	6.05	2.12	J
24	Malic enzyme, hydrogenosomal	EC103587	408	37	71.66	6.06	4.29	C
25	WD40 repeat-containing protein	EC098972	54	12	74.62	6.06	3.05	Z
26	Phosphoenolpyruvate carboxykinase (GTP)	EC107315	48	7	131.17	5.96	4.20	C
27	Peroxidase	EC101256	49	7	130.75	6.01	2.13	R
28	Actin-related protein (similar to 2/3 complex, subunit 1)	EC110654	123	11	39.72	7.14	3.63	Z
29	Citrate synthase	EC104531	137	18	51.89	7.02	2.54	C
30	Aconitate hydratase	EC107496	75	19	95.97	6.19	3.13	CE
31	Mitochondrial aspartate aminotransferase	EC101368	171	36	41.40	-	5.91	E
32	Malate dehydrogenase	EC104397	269	35	45.72	-	4.18	C
33	Actin I	EC111134	27	232	37.80	5.08	-	Z
34	Serine proteinase	EC110565	144	19	36.21	4.98	-	O
35	Actin I	EC102447	42	10	47.86	5.14	-	Z
36	Cysteine proteinase CP1	EC109346	47	4	27.70	5.31	-	O
37	Rho GDP-dissociation inhibitor	EC109901	261	34	22.82	5.62	-	No KOG
38	S-adenosyl-L-homocysteinase hydrolase	EC108366	104	20	59.17	5.62	-	H
39	Methylthioadenosine phosphorylase	EC105195	84	8	25.54	5.80	-	F
40	Fructose-bisphosphate aldolase	EC110582	213	18	41.36	5.88	-	G
41	Vacuolar ATPase B subunit (VatB)	EC107000	87	40	67.53	5.91	-	C

Spot Number[a]	Protein name	Accession number (EST)	MASCOT score[b]	Sequence coverage (%)	M_w (kDa) (obs)	p*I* (obs)	Fold change	Molecular function[c]
42	Ubiquinol cytochrome reductase transmembrane region	EC110722	389	24	24.97	6.04	-	C
43	Histone H2B	EC108883	29	5	22.67	6.05	-	No KOG
44	Oxidoreductase, aldo/keto reductase	EC110349	171	20	33.27	5.96	-	R
45	Putative stress-related protein	EC104139	33	4	44.20	6.10	-	No KOG
46	Transaldolase	EC104642	145	6	23.53	6.12	-	No KOG
47	GAMMA CA3 (GAMMA CARBONIC ANHYDRASE 3)	EC110558	143	14	29.51	6.13	-	R
48	Citrate synthase	EC104531	259	16	51.31	7.54	-	C

[a]Spots are from 2-DE gels. Spot numbers correspond to those indicated in Fig. 1

[b]Mascot score is – 10 x log(P), where P is the probability that the observed match is a random event. Peptide scores > 30 for ESTs database of *Acanthamoeba* spp., indicate identity or extensive homology ($p < 0.05$)

[c]KOG functional categories: (Z) Cytoskeleton; (E) Amino acid transport and metabolism ; (O) Posttranslational modification, protein turnover, chaperones; (G) Carbohydrate transport and metabolism; (R) General function prediction only; (C) Energy production and conversion; (J) Translation, ribosomal structure and biogenesis; (H) coenzyme metabolism; (F) Nucleotide transport and metabolism and (No KOK) protein not related to any KOG category.

4. DISCUSSÃO GERAL

Com o advento de técnicas moleculares modernas, um novo critério para a classificação das espécies do gênero *Acanthamoeba* foi proposto utilizando o sequenciamento do gene 18S rDNA (Gast & Byers, 1995; Stothard *et al.*, 1998). Cada tipo de sequência, com pelo menos 5% de diferença em relação aos outros tipos, além de tornar possível a caracterização genotípica de isolados, também permite inferir relações filogenéticas entre eles. Desse modo, até o momento foram definidos 17 tipos de sequências do 18S rDNA em *Acanthamoeba* spp., sendo o genótipo T4 associado à maioria das infecções humanas, em aproximadamente 90% dos casos de ceratite amebiana (Maghsood *et al.*, 2005; Siddiqui & Khan, 2012; Maciver *et al.*, 2013).

Como parte de um estudo complementar da caracterização de isolados de *Acanthamoeba* spp. obtidos a partir de amostras de água de piscinas em clubes de Porto Alegre, os genótipos de 13 isolados foram identificados molecularmente, utilizando o sequenciamento do 18S rDNA. A amplificação de fragmentos específicos do gênero, região ASA.S1 (aproximadamente 500 pb), utilizando os primers JDP1 e JDP2 foi bem sucedida para todos os isolados analisados. O alinhamento das sequências com o banco de dados nucleotídicos do NCBI (GenBank), através do programa BLASTn, permitiu a classificação dos isolados com identidade igual ou superior a 95%, com sequências de

isolados pertencentes aos genótipos T3, T4 e T5, em nove (69,2%), três (23,1%) e um (7,7%) isolado, respectivamente (Caumo & Rott, 2011).

A diversidade genotípica do gênero *Acanthamoeba* é esperada e suspeita-se que estas formas diversas podem acupar nichos específicos (Maciver *et al.*, 2013). Os grupos genotípicos T3, T4 e T5 são frequentemente relacionados a amostras de água e de poeira. Observa-se que isolados pertencentes ao genótipo T5 são os mais representados em locais de água doce, provavelmente mais adaptados ao potencial osmótico e podem melhor consumir bactérias neste ambiente (Maciver *et al.*, 2013). No presente trabalho, o genótipo T5 foi o mais representado entre os isolados de água de piscinas caracterizados, fato também observado no trabalho realizado por Alves *et al.* (2012), que dentre 19 isolados obtidos a partir de água de piscinas na cidade de Brasília e caracterizados genotipicamente, oito foram classificados como pertencentes ao genótipo T5, seis relacionados ao genótipo T4 e um ao genótipo T2/T6, os outros quatro isolados foram classificados apenas o gênero. Em trabalho realizado por Carlesso *et al.* (2010), isolados obtidos de amostras de biofilmes de ambiente hospitalar foram caracterizados como pertencentes ao genótipo T5. Uma predominância do genótipo T5 também foi evidenciada em amostras ambientais (solo e água) e de lentes de contato nas Filipinas (Rivera & Adao, 2008; Rivera & Adao, 2009). Vários casos de ceratite amebiana e infecções disseminadas por *Acanthamoeba* spp. pertencentes ao genótipo T5, também já foram descritos (Spanakos *et al.*, 2006; Barete *et al.*, 2007; Ledee *et al.*, 2009).

Ceratite amebiana é considerada uma síndrome relacionada à água causada por *Acanthamoeba* spp. e o genótipo T4 é o mais comumente relatado entre os isolados

de ceratite amebiana (Marciano-Cabral & Cabral, 2003; Booton *et al.*, 2005). Este genótipo também é o mais comum em amostras ambientais (Booton *et al.*, 2004).

O genótipo T3 é menos prevalente no ambiente quando comparado aos genótipos T4 ou T5 (Ledee *et al.*, 2009). De acordo com Edagawa *et al.* (2009), este genótipo geralmente é considerado como não patogênico. Desde o primeiro caso de ceratite amebiana associado ao genótipo T3 no Reino Unido (Ledee *et al.*, 1996), poucos casos foram relatados por *Acanthamoeba* spp. pertencentes a este genótipo (Stothard *et al.*, 1998; Zhang *et al.*, 2004; Nagyová *et al.*, 2010). A análise filogenética de 37 sequências 18S rDNA de isolados de *Acanthamoeba* spp. obtidos a partir de amostras de água de rios e de estações de tratamento de água no Japão mostrou a distribuição dos genótipos T3 (56,8%), T4 (13,5%), T5 (8,1%) e T13 (18,9%) (Edagawa *et al.*, 2009). Um padrão semelhante foi documentado em Hong Kong, onde isolados do genótipo T3 foram caracterizados em amostras de água coletadas nas casas de indivíduos com ceratite amebiana (Booton *et al.*, 2002).

Além da diferenciação taxonômica, a relação entre taxonomia e patogenicidade dos isolados tem sido estudada. Observa-se que algumas espécies ou genótipos possuem potencial em causar dano ao hospedeiro, enquanto outros não. Por outro lado, isolados de uma mesma espécie (ou de grupos genotípicos semelhantes) podem causar dano diferencial em culturas de células, em cobaias e humanos, confirmando a existência de uma forte plasticidade fenotípica nestes protozoários (Koehsler *et al.*, 2009; Panjwani, 2010).

Trofozoítos de *Acanthamoeba* spp. são infectivos para uma variedade de hospedeiros mamíferos e podem provocar infecções em humanos, como resultado da complexa interação patógeno-hospedeiro e o ambiente. Grande parte dos danos

causados por trofozoítos em infecções da córnea ou cérebro humano é resultante de vários fatores de virulência e poucos são conhecidos a nível molecular. Estudos proteômicos abrangentes são úteis para a elucidação de tais fatores, a partir da identificação de proteínas envolvidas na interface patógeno-hospedeiro. Para analisar e determinar estes fatores, o presente trabalho realizou estudos proteômicos de trofozoítos de um isolado ambiental de *A. polyphaga* (ATCC 30872), pertencente ao grupo genotípico T4 e obtido de um lago de água doce.

Embora *A. polyphaga* ainda não tenha o genoma sequenciado, a similaridade genética entre as espécies de *Acanthamoeba*, juntamente com os avanços nas tecnologias de espectrometria de massa e softwares para a identificação de proteínas permitiram a identificação eficiente de proteínas de *A. polyphaga*. No presente trabalho, nós combinamos estratégias experimentais complementares (2DE-MS/MS e LC-MS/MS) para analisar o proteoma de trofozoítos de *A. polyphaga*, representando o maior conjunto de dados proteômicos de *Acanthamoeba* spp. até o momento. Estas análises permitiram também estimar quantitativamente a expressão das proteínas resolvidas nos géis 2-DE, para futuros estudos comparativos (entre cisto e trofozoítos, ou entre isolados virulentos e não virulentos, por exemplo). Uma abordagem imunoproteômica de 2-DE imunoblot foi também utilizada na identificação de proteínas antigênicas, revelando potenciais alvos para abordagens imunodiagnósticas e terapêuticas para infecções amebianas por *Acanthamoeba* spp.

As análises baseadas em 2-DE permitiram a resolução de cerca de 370 spots proteicos na faixa de pH de 3-10, a disponibilização do mapa proteômico e a identificação por LC-MS/MS do repertório de proteínas expressas por formas invasivas de *A. polyphaga*. A presente análise proteômica forneceu uma base para os estudos

prospectivos, comparativos e funcionais de proteínas *A. polyphaga* envolvidos nos mecanismos moleculares fundamentais relevantes para o desenvolvimento, sobrevivência e patogenicidade deste patógeno.

As análises por gel 2-DE mostraram evidências de processamento pós-tradução para várias proteínas de trofozoítos de *A. polyphaga*. Modificações pós-traducionais modulam a atividade da maior parte das proteínas eucarióticas e podem determinar a sua localização, o volume, e as interações com outras proteínas (Mann & Jensen, 2003). As variantes proteicas detectadas ou isoformas podem ser o resultado de modificações pós-traducionais biologicamente importantes, variando de modificações químicas à clivagem proteolítica (Ambatipudi *et al.*, 2006). Informações sobre as modificações pós-traducionais de proteínas em *Acanthamoeba* spp. é escassa, mas os nossos resultados indicam que esses fenômenos podem ser frequentes. Entre as proteínas identificadas com aparentes modificações pós-traducionais, encontramos proteína de choque térmico HSP80, gliceraldeído-3-fosfato desidrogenase (GAPDH), a chaperona DnaK, tiorredoxina peroxidase, transaldolase, citrato sintase, enolase, a proteína 14-3-3, actina I , beta-tubulina e S-adenosil-L-homocisteinase. O significado biológico de modificação pós-traducional varia de acordo com o tipo de modificações e em particular de proteínas. Por exemplo, a enzima GAPDH pode ser um alvo de diversas modificações covalentes e tem sido implicada em muitas atividades não relacionadas com a glicólise, tais como a fusão da membrana, a ligação a proteínas hospedeiras e de transdução de sinal (Sirover, 2005). Alvarez *et al.* (2007) relataram em estudo de *E. histolytica*, que a GAPDH e cisteína sintase são proteínas que podem desempenhar um papel importante na sobrevivência da ameba ou na interacção com as células ou moléculas do hospedeiro, tal como ocorre em outros organismos.

Estratégias baseadas em 2-DE e LC-MS/MS são utilizadas amplamente na literatura no estudo dos mais diversos organismos. Bouyer *et al.* (2009) realizaram eletroforese em gel bidimensional para comparar a expressão diferencial de proteínas de trofozoitos e cistos de *A. castellanii*. Cinco das proteínas identificadas (proteína tumoral, actoforina, o factor de elongação 2, proteína de choque térmico 70, frutose bifosfato aldolase e enolase) foram comuns às proteínas ortólogas identificadas na análise do proteoma de trofozoitos *de A. polyphaga* descrito neste trabalho. Dentre outros protozoários, análises proteômicas das proteínas expressas pelos cistos de *E. histolytica* foram realizadas, um total de 417 proteínas não redundantes foram identificadas utilizando técnicas mais sensíveis de espectrometria de massa, que os utilizados no presente estudo (Ali *et al.*, 2012).

Enquanto em nosso estudo, o mapa proteômico de referência de *A. polyphaga* incluiu 136 proteínas identificadas de 370 spots detectados, o proteoma de referência de *Leishmania* (*Viannia*) *braziliensis* apresenta 101 proteínas que representam 75 proteínas identificadas (Cuervo *et al.*, 2007). Os mapas proteômicos de referência de *Trichomonas vaginalis* com diferentes faixas de pH (pH 3-10, pH 4-7, pH 6-11), tiveram um total de 247 spots resolvidos, representando 164 proteínas diferentes (Huang *et al.*, 2009).

A análise comparativa de proteínas expressas por trofozoítos de *A. polyphaga* entre isolados com virulência atenuada por longo tempo de cultura *in vitro* e com recuperação da virulência após infecção em rato, resultou na evidência de expressão diferencial de alguns grupos de proteínas. Pelo menos 48 proteínas diferencialmente expressas foram identificadas, sugerindo uma significativa variabilidade entre os perfis proteicos do mesmo isolado sob duas condições. Talvez

algumas das proteínas não são necessariamente relacionadas a patogenicidade, entretanto alguns fatores envolvidos na virulência e/ou patogenicidade foram identificados como super expressos no isolado virulento (proteínas de choque térmico, proteínas antioxidantes e proteases). Os resultados gerados pelo estudo comparativo ainda estão em fase de análise e provavelmente novas moléculas envolvidas na virulência de *Acanthamoeba* spp. serão identificadas.

No presente trabalho, a realização de imunoblots com soro de ratos infectados com *A. polyphaga* permitiu a identificação de dez proteínas antigênicas (HSP70, chaperonina GroL, hydroxymethylglutarylCoA, inibidor de dissociação GDP, mitocondrial aspartate aminotransferase, RhoGEF domain containing protein, fosfomanomutase, S-adenosyl-L-homocisteina, GAMMA CA3 e ubiquinol citocromo redutase da região transmembrana). A identificação de antígenos de *A. polyphaga* pode levar a um melhor entendimento de quais são as moléculas envolvidas na patogênese (fatores de virulência), que poderão ser utilizadas como marcadores na diferenciação de isolados capazes de causar infecção, bem como fornecer informações importantes para o entendimento das características biológicas do patógeno, mecanismos de evasão da resposta imune, estabelecimento e desenvolvimento da infecção, assim como a identificação de potenciais alvos de drogas para abordagens terapêuticas e desenvolvimento de métodos imunodiagnósticos para infecções por *Acanthamoeba* spp.

5. CONCLUSÕES

O genótipo de 13 isolados ambientais, obtidos da água de piscinas de clubes de Porto Alegre foram identificados a partir do sequenciamento parcial da região 18S do rDNA. A determinação correta da taxonomia é um fator essencial para que se possa chegar a generalizações acerca da ecologia, patogenicidade e epidemiologia de *Acanthamoeba* spp.

Este estudo é a primeira análise proteômica do repertório de proteínas expressas por trofozoítos de *A. polyphaga* e o estudo proteômico mais abrangente realizado para o gênero. O protocolo de 2-DE desenvolvido teve boa reprodutibilidade para extratos proteicos de trofozoítos de *A. polyphaga* e a disponibilidade de sequências ortólogas permitiu a identificação bem sucedida das proteínas expressas por trofozoítos.

O mapa proteômico de *A. polyphaga* (pH 3-10) foi produzido e a sua disponibilidade serviu como base para o estudo proteômico comparativo.

A análise comparativa de proteínas expressas por trofozoítos de *A. polyphaga* entre isolados com virulência atenuada por longo tempo de cultura *in vitro* e com recuperação da virulência após infecção em rato, resultou na evidência de

expressão diferencial de alguns grupos de proteínas.

Proteínas antigênicas de *A. polyphaga* foram identificadas e 9 spots proteicos foram caracterizados, correspondendo a 10 antígenos descritos.

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ANEXOS

Os artigos em anexo, correspondem a trabalhos publicados durante o período de doutoramento em colaboração. Estes artigos referem-se ao isolamento, identificação morfológica e caracterização genotípica de isolados ambientais de *Acanthamoeba* spp.

ANEXO I

Artigo "Potentially pathogenic *Acanthamoeba* isolated from a hospital in Brazil" de autoria de Carlesso AM, Artuso GL, Caumo K, Rott MB., publicado no periódico *Current Microbiology*, v. 60, pp 185-190 no ano de 2010.

A minha colaboração neste trabalho foi na realização das metodologias para genotípagem e identificação dos genótipos, bem como a redação dos resultados referentes à caracterização genotípica.

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Potentially Pathogenic *Acanthamoeba* Isolated from a Hospital in Brazil

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Abstract Studies on free-living amoebae (FLA), has been increased in recent years, especially related to the genus *Acanthamoeba*, because these organisms are widely found in the environment. The present work isolated and characterized this organism from biofilms and dust in hospital environment. 135 samples were collected in 15 different environments in a hospital at the south of Brazil. Thirty-one (23%) isolates were identified as morphologically belonging to the *Acanthamoeba* genus and 10 of these were submitted to temperature and osmotolerance tests as criterion for evaluation of the viability and pathogenicity. The tests indicate that four (40%) of these isolates could be potentially pathogenic because grew at high temperature (40°C) and osmolarity (mannitol 1 M). Some isolates genotypes were determined after ribosomal DNA sequencing. These data revealed that three dust isolates belong to T4, two biofilm isolates to T5 and one dust isolate to T3 genotype. Therefore, *Acanthamoeba* found in the hospital environment represents a risk for people that circulate there.

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Introduction

Free-living amoebae (FLA) of the genus *Acanthamoeba* have been reported to be of medical interest as etiological agents of infection in immunodeficient hosts and keratitis in the contact lens wearer [9, 12]. These protozoa are widely distributed in the environment and commonly found in two forms: the vegetative trophozoite, and the uninucleated cyst form that is able to resist long periods of desiccation and extreme conditions of temperature, pH, and exposure to diverse chemicals [1, 24, 29]. The genus is easily differentiated from other amoebae by the characteristic shape of its cysts. Pussard and Pons [18] classified *Acanthamoeba* into three groups according to size and morphological differences. Besides being the most frequently isolated amoeba, it is estimated that the genus *Acanthamoeba* may be the most common free-living protozoa; this confers to the genus a large ecological importance. This organism is found in the soil, air, and in fresh and salty ocean water. Its primary source of food is bacteria that can be found in great quantities in polluted areas, proliferating greatly in mild temperatures. A hot humid environment also promotes the use of air conditioners in closed rooms, resulting in a proliferation of *Acanthamoeba* on the cooling equipment at a lower temperature [1, 5, 6, 13, 20]. The presence of these protozoa in hospital environment may represent a health risk to workers, patients, and other individuals that circulate in these locations. The objective of this study was to isolate FLA of the genus *Acanthamoeba* from biofilms and dust collected from hospital environments as well as

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identifying their morphological, physiological, and genetic characteristics.

Materials and Methods

Samples

135 samples of dust and/or biofilms were collected for 9 months from 15 locations in environmental of a public hospital in the city of Porto Alegre, RS, Brazil which were: intensive care center (ICC), surgical center (SC), pediatric intensive care unit (PICU), kitchen (K) emergency room (ER), outpatient surgical center (OSC), tile water storage tanks (TWST) situated in the basement, cement water storage tanks (CWST) located outside the hospital, six drinking fountains for general use (WF) located inside the hospital, and a kitchen tap (KT). The climactic data relative to the monthly average temperature was obtained from the weather station (INMET) seoma8@inmet.gov.br Samples were collected using dry sterilized swabs from random spots picked in the chosen environment; such as: the floor, window, and furniture, collection of biofilms from the drinking fountain, water taps, and storage tank. These samples were processed according to Silva and Rosa [26].

Amoebae Culture and Morphological Identification of the Genus *Acanthamoeba* Isolates

For the isolation of amoebae in a monoxenic culture, a 1.5% non-nutrient agar covered by heat-inactivated *Escherichia coli* (ATCC 25922) was used [4]. Before the isolates were axenized in a PYG (2% protease peptone, 0.2% yeast extract, and 1.5% glucose) at 30°C. Identification of the amoeba was based on the type of movement and morphological criteria of the cysts and trophozoites structures proposed by Page [16]. The morphological study of potentially pathogenic FLA belonging to the genus *Acanthamoeba* was completed by observations of the morphological characteristics and measurement of cysts and trophozoites using a micrometric ruler and optical microscope. This analysis was made by observation of translucent structures, fixed, and stained with trichrome according Garcia and Bruckner [7].

Organisms Exflagellation Assays

For the exflagellation assays, the amoebae obtained from monoxenic cultures of the all isolates, were gently scraped and suspended in 10 ml of distilled sterile water in tissue culture flasks (25 cm^3) and incubated at 37°C. The flasks were examined each 30 min, for 4 h, using inverted microscope in order to check the emission of flagella [4].

Tolerance Assays

In order to analyze the effect of osmolarity in the *Acanthamoeba* trophozoite growth, 1.5% non-nutrient agar plates with an overlayer of *Escherichia coli* suspension (ATCC 25922), containing mannitol 0.5 and 1.0 M were used. Plates under the same conditions but with no mannitol were used as growth control. Approximately 10^3 trophozoites were inoculated in the center of the plates and incubated at 30°C for up to 10 days.

For the assay of tolerance to temperature approximately 10^3 trophozoites were inoculated with *E. coli* in the central area of the 1.5% non-nutrient agar plates, and incubated at 37, 40, and 42°C for 10 days. The plates, submitted to 30°C, were used as control in growth assessment.

In order to evaluate the growth of the organisms in both assays, on the tenth day of incubation the cysts and trophozoites were observed in optical microscopy (100×) at about 20 mm from the center of the plate. All assays were carried out in triplicates, and *Acanthamoeba* environmental and clinical strains (ATCC 30010 and ATCC 50492) were used as positive and negative controls, respectively.

Molecular Identification of *Acanthamoeba* Isolates

DNA extractions were performed using 1.0 ml volume of the axenic and/or monoxenic cultures containing 10^2 trophozoite according to the method described by Khan et al. [11] and the concentration evaluated by the method of Sambrook et al. [21]. Primers JDP1 and JDP2 were used to amplify the ASA.S1 region of the gene (*Rns*) coding for the amoeba's nuclear, small subunit ribosomal RNA [22]. The ASA.S1 fragment allows the specific detection of *Acanthamoeba* since it is discriminating for the genus and can be obtained from all known 18S rDNA genotypes [22]. The polymerase chain reaction (PCR) was performed as previously described by Booton et al. [3]. Briefly, the amplifications were carried out in a 50 µl volume containing 20–30 ng of DNA, 0.2 mM dNTPs (Mix, Invitrogen™), 0.4 µM of each oligonucleotide, reaction buffer (50 mM KCl_2, 10 mM Tris–HCl), 1.5 mM of magnesium chloride, and 1 U of Platinum® *Taq* DNA Polymerase (Invitrogen™). The amplification reaction was carried out in a PTC-150 Minicycler MJ Research thermocycler, under the following conditions: 94°C for 1 min, 67°C for 1 min, 72°C for 1 min, and 72°C for 5 min. The amplification product was separated in 2% agarose gel, stained with 0.5 µM/ml ethidium bromide, observed under a UV-light transilluminator.

PCR products were purified using QIAquick® extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions and resolved with an MegaBace 1000 automated sequencer and the sequences were deposited in the GenBank database and are available under the

Table 1 Origin, cyst morphology, accession numbers, and genotype of *Acanthamoeba* isolates/strain under study

Strain/isolate	ATCC number	Source	Genotype	GenBank accession number	Cyst morphology[a]
Acanthamoeba castellanii Neff	30010	Soil	T4		II
Acanthamoeba castellanii	50492	Keratitis (India)	T4		
K8		Dust	T3	GQ999992	I
TWST7		Biofilm	T5	GQ999993	II
TWST9		Biofilm	T5	GQ999994	I
ER8		Dust	T4	GQ999995	III
OSC7		Dust	T4	GQ999996	I
K4		Dust	T4	GQ999997	II
K5, K7		Dust			I
WF147, WF137, WFE2, WF084, WF087, K2, ER7, TWST8, PICU9		Biofilm/dust			II
WFE7, WF196, WF131, WF157, WF081, WF148, WF199, WF146, WF083, WF198, WF143, K9, KT9, CWST9		Biofilm/dust			III

[a] Grouping was performed according to method of Pussard and Pons [18]

accession numbers: GQ999992, GQ999993, GQ999994, GQ999995, GQ999996, and GQ999997 (Table 1). Sequences were uploaded into the Basic Local Alignment Search Tool (BLAST) program of the US National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) to search for the most similar reference sequences.

Results and Discussion

Of the 135 samples collected, 47 (35%) were positive for FLA. From these, 31 (23%) presented as FLA that seems to belong to the genus *Acanthamoeba*. These isolates had trophozoites (Fig. 1) with slow motion, cytoplasm with a hyaline peripheral zone, from which was noted thin acanthopodic projections and the presence of pulsatile vacuoles in the cytoplasm. Cysts (Fig. 1) were characterized by the shape of the endocyst and ectocyst according to Pussard and Pons's [18] classification (Table 1). Our observations are in agreement with Schuster and Visvesvara [24] which reported that the dimension of *Acanthamoeba* trophozoites, range between 15 and 30 μm and the cysts between 10 and 15 μm. Moreover, they also pointed to the presence of double walled and pores on the cysts and acanthopodia on the trophozoites (Fig. 1).

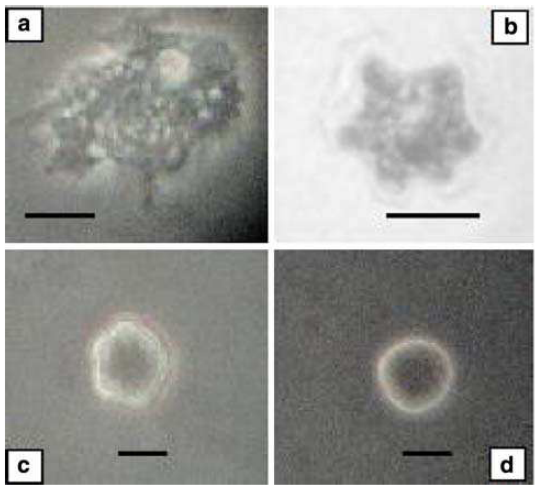

Fig. 1 Trophozoite (**a**) and cysts (**b**–**d**) of the isolates under study. The *bars* represent 10 μm

The exflagellation assay was negative for all isolates obtained of amoebae in monoxenic culture, indicating the inexistence of any *Naegleria fowleri* isolate.

The location, where FLA possibly belonging to the *Acanthamoeba* genus, were isolated and the percentages on the total number of samples collected ($n = 135$) are shown in Table 2. Silva and Rosa [26] isolated FLA from dust in all the hospital environments that they analyzed, being 45.5% of the isolates belonging to the genus *Acanthamoeba*.

During the collection period (July to March), January was the month that shown the highest number of positive findings for the genus *Acanthamoeba* (9 isolates). The average temperature for this month was 26.8. Figure 2 shows the average temperatures, the months in which samples were collected, and the number of positive samples containing *Acanthamoeba*. Few studies report the seasonality of FLA in environmental samples. However, some authors describe the hypothesis that *Acanthamoeba* is more prevalent in warmer environments or during the summer months [30]. In contrast to this, other authors affirm that the production of highly resistant cysts by these protozoa could explain why some of these studies did not show significant difference in the number of amoebae isolates in different

Table 2 Number of positive samples probably of the genus *Acanthamoeba* in a hospital environment and percentages on the total collected samples

Hospital environment	Number of isolates possibly belonging to the genus *Acanthamoeba*	% on the total number of samples collected ($n = 135$)
WF	16	11.85
OSC	1	0.74
K	6	4.44
KT	1	0.74
ER	2	1.48
TWST	3	2.22
CWST	1	0.74
PICU	1	0.74
Total	31	22.96

WF water fountain, *OSC* outpatient surgical center, *K* kitchen, *KT* kitchen tap, *ER* emergency room, *TWST* tile water storage tanks, *CWST* cement water storage tanks, *PICU* pediatric intensive care unit

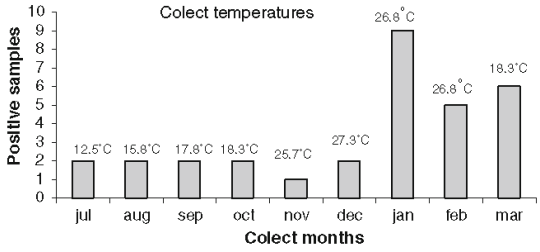

Fig. 2 Collection month and average temperatures versus number of positive samples of *Acanthamoeba*

seasons of the year [6]. In 1993, Rivera et al. [19] studied the presence of *Acanthamoeba* in the water of 12 swimming pools and 13 jacuzzis and reported an increased detection during the summer months and a considerable decrease in the winter months.

In our study, only 10 isolates identified as belonging to the genus *Acanthamoeba* were submitted to different temperatures (37, 40, and 42°C) and osmolarities (0.5 and 1 M) for evaluation of viability and pathogenic potential. The others were not evaluated because the samples were so contaminated interfering in the tests. From this group, all isolates grew at 37°C, 7 grew at 40°C, and no growth was observed at 42°C (Table 3). Several authors refer the thermal tolerance as being a factor that can determine pathogenicity in an isolate [6, 15]. In 2002, Schuster used clinical samples such as cerebrospinal fluid, brain tissue, scrapings from skin, and corneal scrapings to isolate potentially pathogenic FLA belonging to the genus *Acanthamoeba*. From these samples, isolation in non-nutrient agar occurred in 1–2 days at 37°C, confirming that at this temperature clinical isolates of *Acanthamoeba* grows readily. In another

Table 3 Behavior of *Acanthamoeba* isolated from dust and biofilm at different temperatures and osmolarities

Isolates	Temperature growth			Mannitol growth	
	37°C	40°C	42°C	0.5 M	1.0 M
WF147	+	+	−	+	+
OSC7	+	+	−	+	+
K4	+	+	−	+	−
K5	+	+	−	+	+
K8	+	+	−	+	+
ER7	+	−	−	+	−
ER8	+	−	−	+	−
TWST7	+	+	−	+	−
TWST8	+	−	−	+	−
TWST9	+	+	−	+	−

+ viability or growth, − absence of growth

work, Schuster and Visvesvera [24] reported that increasing in global warming concerning regarding the dispersion of parasitic diseases. For the FLA in the soil, raising the temperature could increase the appearance of thermotolerant species, which is better suited when they invade humans or animals. However, these authors state that thermotolerance is not necessarily synonymous of pathogenicity or virulence for the growth of FLA.

From the 10 isolates of genus *Acanthamoeba* assessed, all have shown growth at mannitol 0.5 M and 4 showed growth in high osmolarity (mannitol 1 M) (Table 3). Therefore, the tests indicate that four (40%) of these isolates could be potentially pathogenic because grew at high temperature (40°C) and osmolarity (mannitol 1 M).

The growth variation of an isolate in high osmolar concentration and temperature can be related to virulence, since a potentially pathogenic isolate can be considered more virulent or less depending on their capacity for adaptation and viability in the tissues of the host, factors related to development of pathology. Some studies have pointed out that *Acanthamoeba* pathogenic isolates have shown growth to increased temperatures and osmolarity [10] and that these physiological determinants can be used in the differentiation and previous characterization of the environmental and clinical isolates correlated to assays of cytopathic effects [11].

Some of the isolates obtained in our study were identified as belonging to the genus *Acanthamoeba* according to the morphological criteria proposed by Page [16], and by literature data and were confirmed by PCR. The difficulty in axenizing positive *Acanthamoeba* samples is a frequent report in the literature [8, 10]. The main interferents were fungi and bacteria. Furthermore, it is known that there are some strains of *Acanthamoeba* are difficult to multiply in PYG medium, requiring the most enriched [17, 23, 25].

Ten axenic or monoxenic isolates (WF147, OSC7, K4, K5, K8, ER7, ER8, TWST7, TWST8, and TWST9) were submitted to PCR. The others, were not evaluated because the samples were so contaminated and DNA quality was very poor. DNA was extracted and amplified using specific oligonucleotides [22]. An amplification product of 423- to 551-bp was obtained for all isolates using JDP1 and JDP2 primers.

Six genotypes were identified from ten dust and biofilm isolates. Three of the ten isolates were genotype T4 while two were genotype T5 and one was genotype T3 (Table 1). The three dust isolates, ER8, OSC7, K4, detected in this study were 96, 99, and 98%, respectively, similar to the *Acanthamoeba* sp. T4 sequence chosen as reference (accession number GQ342606). The two biofilm isolates, TWST9 and TWST7, were 96 and 98%, respectively, similar to *Acanthamoeba* sp. genotype T5 sequence chosen as reference (accession number FJ422546). The dust isolate K8 was 96% similar to the *Acanthamoeba* sp. T3 sequence chosen as reference (accession number FJ042644). The three genotypes of *Acanthamoeba* identified in the study are commonly found in environmental samples reported in other researcher's studies and have established T4 genotype as predominant group responsible for *Acanthamoeba* infections in humans [9, 14]. Virulent strains of *Acanthamoeba* species are usually identified as T4 [28, 31]. Isolates of T5 genotype are usually free-living, although there are cases where this genotype has been isolated in patients with *Acanthamoeba* keratitis [27] and disseminated acanthamoebiasis [2].

In conclusion, the spots analyzed shelter patients at risk and these amoebae could represent a potential danger to the development of diseases with difficult diagnosis and high mortality, beyond to convey pathogens responsible by nosocomial infections. Taking into account that in Brazil, there have been a few earlier studies on the presence of FLA in hospital environments, our results are so important since indicate the high pathogenic potential of these environments, especially the water fountains and kitchen, which can be major sources of hospital infection, in view of the number of patients, relatives, and professionals who use these services. It is essential that committees of hospital infection direct efforts to implement measures of hygiene and disinfection more efficient to control these agents, especially in the warmer seasons of the year and in those environments where the biofilm is abundant.

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ANEXO II

Artigo “Prevalence of *Acanthamoeba* from tap water in rio grande do Sul, Brazil” de autoria de Winck MA, Caumo K, Rott MB., publicado no periódico *Current Microbiology*, v. 63, pp 464-469 no ano de 2011.

A minha colaboração neste trabalho foi na orientação para a realização da parte experimental para genotipagem dos isolados e análise dos dados gerados, assim como a revisão do manuscrito referente a caracterização genotípica.

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Prevalence of *Acanthamoeba* from Tap Water in Rio Grande do Sul, Brazil

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Abstract A total of 136 samples of tap water were collected from state and municipal schools between March and November 2009. The samples were filtered through cellulose nitrate membranes that were seeded at non-nutrient agar 1.5% containing an overlayer of *Escherichia coli* suspension. Thirty-one (22.79%) tap water samples investigated were found positive for free-living amoebae (FLA). From these, 13 presented as FLA that seems to belong to the genus *Acanthamoeba.* All samples of FLA were cloned and identified as belonging to the genus *Acanthamoeba* by the morphology of cysts and trophozoites and by PCR using genus-specific primers that amplify the ASA.S1 region of 18S rDNA gene. Physiological tests of thermotolerance and osmotolerance were used to evaluate the pathogenicity of the isolates. The sequencing analysis by comparing the sequences submitted to GenBank, showed genotype distribution into groups T2, T2/T6, T6, and T4. In tests of thermotolerance and osmotolerance, 50% of the isolates had a low pathogenic potential. The results indicated the presence of *Acanthamoeba* in tap water in Rio Grande do Sul, Brazil, revealing its importance and the need for more epidemiological studies to determine their distribution in the environment and its pathogenic potential.

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Introduction

Free-living amoebae (FLA) of *Acanthamoeba* genus are widely distributed in the environment and may become pathogenic amoeba to man. They have been isolated from soil, saltwater, freshwater, humans, and several domestic and wild animals [29]. These protozoa resist to long periods of desiccation and extreme conditions of temperature, pH, and exposure to various chemicals [35, 40, 44]. Several species of *Acanthamoeba* are clinically significant, with the potential to cause a corneal infection termed *Acanthamoeba* keratitis (AK); some strains can cause granulomatous amoebic encephalitis (GAE), a fatal brain infection [17, 28, 40, 47].

Human amoebic keratitis infection occurs through contamination from rinsing contact lenses in non-sterile water, or through wearing contact lenses while bathing or swimming in a no-disinfected aquatic environment [19, 30, 34, 37, 40, 41].

The identification of the genus *Acanthamoeba* is usually based on the trophozoites and cysts morphology especially on the cysts double wall. *Acanthamoeba* isolates can be separated into distinct morphological groups known simply as I, II, and III [36]. The taxonomy and classification of these protozoa are continuously under revision, following the successful application of molecular techniques [3, 5, 43]. Evolutionary studies have led to the identification of at least 16 genotypes (T1–T16) based on rRNA gene sequencing [5, 9, 13, 26, 43].

Human infections by *Acanthamoeba* remain underinvestigated in Brazil, however, some cases of keratitis and studies of clinical and environmental isolates have been made [1, 6, 8, 27]. The presence of these protozoa in tap water may represent a health risk to individuals. The objective of this study was to isolate FLA of the genus

Acanthamoeba from tap water as well as identifying their morphological, physiological, and genetic characteristics.

Materials and Methods

Sample Collection

A total of 136 tap water samples were collected from state and municipal schools of Rio Grande do Sul (RS), Brazil. Samples were collected between March and November 2009.

Isolation of FLA

Water samples (~1000 ml) were filtered through cellulose nitrate filter (pore size 3.0 μm; diameter, 47 mm; Sartorius Biotech). Each filter was transferred to an 82-mm wide plate of 1.5% non-nutrient agar (NNA) containing an overlayer of an *Escherichia coli* (ATCC25922) suspension that had been heat inactivated (for 2 h at 56°C). The plates were sealed with Parafilm® (SPI Supplies, West Chester, PA) and then incubated at 30°C for up to 15 days. Each plate was examined daily under a light microscope (at 100×) to check the presence of FLA. All positive samples were cloned by the method of dilution, where only one microorganism is seeded in each isolate.

Morphological Identification of *Acanthamoeba* Isolates

The trophozoites and cysts of the FLA isolated from tap water were morphologically assessed [40], with particular attention paid to the double wall of the cysts [33, 36]. The sizes of the cysts and trophozoites were estimated from trichome-stained smears [12], using a calibrated ocular micrometer.

Molecular Identification of *Acanthamoeba* Isolates

The total DNA in sample of each FLA-positive culture (containing 10^6 trophozoites) was extracted, as described by Salah and Iciar [38]. Primers JDP1 and JDP2 were used to amplify the ASA.S1 region of the gene (Rns) coding for the amoeba's nuclear small-subunit ribosomal RNA [39]. The polymerase chain reaction (PCR) was performed as described by Booton et al. [4]. Briefly, the amplifications were carried out in a 50 μl volume containing 20–30 ng of DNA, 0.2 mM dNTPs (Mix, Invitrogen™), 0.4 μM of each oligonucleotide, reaction buffer (50 mM KCl_2, 10 mM Tris–HCl), 1.5 mM $MgCl_2$, and 1 U of Platinum® Taq DNA Polymerase (Invitrogen™). The amplification reaction was carried out in a PTC-150 Minicycler MJ Research thermocycler, under the following conditions: 94°C for 1 min, 67°C for 1 min, 72°C for 1 min, and 72°C for 5 min. The amplification product was separated in 1.5% agarose gel, stained with 0.5 μM/ml ethidium bromide, and observed under UV-light transilluminator.

PCR products were purified using a QIAquick® extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions, and resolved with a MegaBace 1000 automated sequencer.

In order to classify the *Acanthamoeba* isolates of this study, 13 sequences of 18S rDNA gene were uploaded into the Basic Local Alignment Search Tool (BLAST) program of the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) to search for the most similar sequences.

Tolerance Assays

All the tolerance assays were carried out in triplicate, with the environmental (Neff) and clinical (T4) strains of *A. castellanii* used as reference examples of nonpathogenic and pathogenic isolates, respectively.

Osmotolerance

In order to investigate the effect of osmolarity on the growth of *Acanthamoeba* trophozoites of each isolate, trophozoites were transferred (at 10^3 trophozoites/plate) to the center of fresh plates of 1.5% non-nutrient agar containing no mannitol (as a control) or 0.5 or 1.0 M mannitol each, with the usual overlayer of *E. coli* suspension. The plates were incubated at 30°C for 10 days and the growth was evaluated. For this, the number of trophozoites or cysts seen, about 20 mm from the center of each plate, in five microscope fields at 100× were counted, and the presence of growth and no growth being scored − and + , respectively [8].

Thermotolerance

For the assay of tolerance to temperature, plates were inoculated as for the osmotolerance assay and incubated at 30 (as a control), 37, or 42°C for 10 days. Growth at the end of this incubation was assessed in the same way as for the osmotolerance assays.

Results and Discussion

This is the first study of the occurrence of *Acanthamoeba* in tap water samples from state and municipal schools of Rio Grande do Sul, Brazil. Of the 136 tap water samples analyzed, 31 (22.79%) were found positive for FLA. From these, 13 presented as FLA that seems to belong to the

Table 1 Genotypic characterization of isolates found in tap water in RS-Brazil

Isolate	GenBank accession	Genotype	Genbank accession	Reference sequences[a] Identity (%)
TW78	JF718254	T2	GU597010	99
TW79	JF718255	ND	ND	ND
TW80	JF718256	T2	GU597006	99
		T6	GU573874	99
TW81	JF718257	T2	GU597010	100
TW82	JF718258	T2	GU597010	99
TW83	JF718259	T2	GU597011	98
TW84	JF718260	T2	GU597006	99
		T6	GU573874	99
TW95	JF718261	T4	FJ422527	100
TW109	JF718262	T2	GU597010	99
TW119	JF718263	T2	GU597006	97
		T6	GU573874	97
TW122	JF718264	T6	GU573874	98
TW125	JF718265	T2	GU597006	99
		T6	GU573874	99
TW132	JF718266	T4	EU168082	98

[a] Percentage of identity and access number compared to GenBank sequences of isolates of this study; ND: *Acanthamoeba*, but genotype not determined

genus *Acanthamoeba*. The presence of *Acanthamoeba* in tap water can be explained by the resistance of the cysts of this genus to water chlorination.

These isolates had trophozoites with slow motion, cytoplasm with a hyaline peripheral zone, from which was noted thin acanthopodic projections and the presence of pulsatile vacuoles in the cytoplasm. Our observations are in agreement with Schuster and Visvesvara [40] which reported that the dimension of *Acanthamoeba* trophozoites, range between 14.3 and 18.8 μm the cysts between 11.5 and 16 μm (Table 1). Cysts were characterized by the shape of the endocyst and ectocyst according to Pussard and Pons's [36] classification. Eight strains were classified as belonging to group II (TW79, TW80, TW81, TW82, TW95, TW119, TW122, and TW125), and two isolates to group III (TW83 and TW84). The *Acanthamoeba* most commonly isolated from the environment tend to be of morphological group II [32] but this group also includes the *Acanthamoeba* species responsible for most cases of granulomatous encephalitis and keratitis [43].

The 13 isolates obtained in this study, all morphologically characterized as *Acanthamoeba*, were confirmed by means of PCR. According to Mathers et al. [31] and Tsvetkova et al. [45], PCR offers a highly sensitive and specific method for the detection and identification of FLA, including those in clinical samples. The usefulness of morphology alone as a taxonomic criterion for the identification of FLA appears limited [46], especially as even a cloned isolate can produce cysts of varying morphology [33]. To complement the older, more conventional tests for FLA differentiation, PCR employing genus-specific primers based on 18S rDNA sequences have been used to identify *Acanthamoeba* [31].

It's sources of tap water, for drinking, washing, or cooking, are clearly important in the exposure of humans to *Acanthamoeba* and, probably, for the development of amoebic keratitis [25]. Overall, there is a clear need for more detailed knowledge about the distribution of *Acanthamoeba* in different environments, genotypes characterization, and their direct and indirect virulence factors. These findings will be useful to understand the basis of differential distribution of genotypes in tap water which should allow the identification of their properties contributing to the development of therapeutic measures against these pathogens.

As part of a more comprehensive study examining the genotype of *Acanthamoeba* spp. isolates obtained from tap water from state and municipal schools of Rio Grande do Sul, Brazil, we identified *Acanthamoeba* genotypes molecularly, using 18S rDNA sequencing of 13 isolates. Amplification of genus-specific amplimers ASA.S1 with primers JDP1 and JDP2 was successful in all 13 isolates tested. Amplimers ASA.S1 (approximately 500 bp) were obtained from all isolates. The 13 18S rRNA sequences from the *Acanthamoeba* isolates were aligned with *Acanthamoeba* sequences retrieved from GenBank. Our results revealed that *Acanthamoeba* isolates from tap water belong to genotypes T2, T4, and T6 (Table 1).

Six of the 13 sequences revealed a strict correspondence with the available sequences of the T2 genotype. The percentage of identity between the obtained sequences and the six T2 sequences chosen as reference ranged from 97 to

Table 2 Behavior of *Acanthamoeba* isolated from tap water at different temperatures and osmolarities

Isolates	Temperature growth		Mannitol growth	
	37°C	42°C	0.5 M	1.0 M
TW79	+	−	+	−
TW80	+	−	+	+
TW81	+	−	+	−
TW82	+	−	+	−
TW83	+	−	+	−
TW84	+	−	+	+
TW95	+	+	+	−
TW119	+	−	+	+
TW122	+	−	+	−
TW125	+	−	+	+
A. castellanii[a]	+	+	+	+

[a] Strain reference (ATCC 50492), + viability or growth, − absence of growth

100%. Four isolates are related to *Acanthamoeba* genotype T2/T6 sequences chosen as references, ranged from 97 to 99% identity. One of the 13 isolates analyzed (TW122) showed a strict correspondence with the deposited sequences for genotype T6, with 98% identity with the T6 sequence chosen as reference. And two isolates analyzed (TW95 and TW132) showed a correspondence with the deposited sequences for genotype T4, the most commonly reported among keratitis isolates [5, 28]. However, there are several known cases of eye infection associated with the following sequence types: T2 [26], T3 [23, 43], T5 [24, 42], T6 [48], T11 [18], and T15 [10].

This study of the distribution of *Acanthamoeba* genotypes from tap water in Brazil, indicated genotype T2 and T6 as the most common among the sequences analyzed and two isolates belonged to genotype T4. A study examining the presence of *Acanthamoeba* spp. in Brazilian water sources, of the 19 tap water samples from the metropolitan area of São Paulo city (São Paulo State, Brazil), showed that two samples from the same school were positive for *Acanthamoeba* and one was identified with the genotype T4 species [27]. In Rio Grande do Sul, Brazil, a study about the frequency of *Acanthamoeba* isolated from water in 13 swimming pools revealed that nine were genotype T5, three isolates belonged to genotype T4 and an isolated to the T3 [7].

In study conducted by Maghsood et al. [26], *Acanthamoeba* genotypes were determined for 13 keratitis isolates and 12 water isolates from Iran. The majority of these 12 environmental isolates tested belonged to T2 (58.3%), followed by T4 isolates (33.3%) and clinical isolates, 61.5% belonged to genotype T4, 15.3% belonged to T3, and 23% belonged to T2. The T2 and T6 genotypes have also been isolated previously from spring water samples in the Taiwan, where genotype T6 was more commonly isolated [16].

Previous studies have suggested that many *Acanthamoeba* isolated from tap water and seawater sources might possess some pathogenic ability [11, 21, 25, 27].

In tests carried out in a study of tap water samples developed by Magliano et al. [27] in Brazil, the pathogenic potential of an isolate belonging to T4 group was determined from the secretion of proteolytic enzymes. Lorenzo-Morales et al. [25] reported the presence of potentially pathogenic in FLA of genus *Acanthamoeba* in tap water and sea water sources through temperature and osmotolerance assays. Our results of the tolerance assays are summarized in Table 2. Ten of the *Acanthamoeba* isolates investigated grew at 37°C and, at 30°C, with 0.5 M mannitol. The isolates that either grew at 42°C but not, at 30°C, with mannitol at 1 M (TW95), or grew with mannitol at 1 M but did not grow at 42°C (TW80, TW84, TW119, and TW125), total of 50% were considered to have low pathogenic potential, and the remaining isolates (TW79, TW81, TW82, TW83, and TW122)—which did not grow at 42°C or, with mannitol at 1 M—were considered probably nonpathogenic.

The in vitro growth of an *Acanthamoeba* isolate under relatively high osmotic stress or at a relatively high temperature can be related to virulence, since virulence is at least partially associated with an isolate's capacity to adapt and remain viable in the tissues of a mammalian host [19, 20]. Some studies have pointed out that *Acanthamoeba* pathogenic potential isolates have shown growth to increased temperatures and osmolarity and that these physiological determinants can be used in the differentiation and previous characterization of the environmental and clinical isolates correlated to assays of cytopathic in tissue cultures effects and in vivo infections [14, 19, 22].

Although 60% of the tap-water isolates of *Acanthamoeba* investigated by Hong et al. [15] were osmotolerant and thermotolerant, only 20% showed the serino-protease expression that was taken as a marker of virulence. Even isolates that are not directly pathogenic to humans may have epidemiological relevance, however, as they can be the carriers of pathogenic bacteria such as *Pseudomonas aeruginosa* and *Legionella pneumophilla* [2].

All 13 *Acanthamoeba* isolates investigated grew at 37°C. Strains of *Acanthamoeba* which can infect humans and other mammals must be capable of surviving at temperatures of 37°C and slightly higher body temperatures. Different species may be thermotolerant but nonpathogenic, making thermotolerance a necessary but insufficient condition for the determination of pathogenicity. Thermotolerance is also less indicative for species that infect the human cornea. Corneal temperature is around 32–35°C

facilitating the ability of *Acanthamoeba* to colonize the surface of the cornea [40].

Then, our data suggest that the prevalence of *Acanthamoeba* in tap water and the resistance of the organism to treatment to obtain drinking water constitute an important factor to the contamination when associated with the hygiene of contact lenses by users. According to Kilvington et al. [21], water storage tanks may promote *Acanthamoeba* colonization in domestic tap water increasing the risk of contamination in contact lense users and consequently the risk of AK.

In conclusion, *Acanthamoeba* were detected in 9.5% of the tap water confirming the wide-spread distribution of these FLA in the environment and the risks associated the contamination of contact lens wearers. The characterization of the *Acanthamoeba* isolates collected, in thermo and osmotolerance assays, indicated that some of the isolates are potentially keratopathogenic.

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ANEXO III

Artigo “Isolation and genotyping of free-living environmental isolates of *Acanthamoeba* spp. from bromeliads in Southern Brazil” de autoria de Landell MF, Salton J, Caumo K, Broetto L, Rott MB., publicado no periódico *Experimental Microbiology*, no ano de 2013.

A minha colaboração neste trabalho foi de orientação de aluno de iniciação científica para a realização do isolamento e caracterização morfológica, bem como axenização dos isolados, auxílio na descrição dos resultados e discussão do manuscrito.

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Research Brief

Isolation and genotyping of free-living environmental isolates of *Acanthamoeba* spp. from bromeliads in Southern Brazil

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HIGHLIGHTS

- First occurrence of *Acanthamoeba* similar to genotypes T2/T6, T4 and T16 associated with bromeliads.
- All isolates grew at 37 C and seven grew in media with 0.5 M mannitol but not to 1 M.
- Identification of *Acanthamoeba* isolates from phylloplane bromeliads.

GRAPHICAL ABSTRACT

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ABSTRACT

Species of *Acanthamoeba* are frequently isolated from distinct environmental sources such as water, soil, dust and air. They are responsible to cause infections and disease in humans and animals. In addition, *Acanthamoeba* sp. are considered an important reservoir of bacteria, virus and fungi, which act as "Trojan horses" to protect these microorganisms of harsh environmental conditions. In this study, nine *Acanthamoeba* isolates from bromeliads phylloplane were identified based on the morphology of cyst and trophozoite forms. The genotype level was accessed by the sequence analysis of *Acanthamoeba* small-subunit rRNA gene. Genotypic characterization grouped five isolates in the genotype T2/T6, three in the T4 genotype and one in the genotype T16. The results obtained indicate that the genotype T2/T6 is common on phylloplane. To predict the pathogenic potential of the *Acanthamoeba* isolates, thermo and osmotolerance assays were employed, although all isolates were capable of surviving at temperatures of 37 °C, other tests will be conducted in the future to determine the potential pathogenic of the isolates. Altogether, our results revealed the importance of the presence of *Acanthamoeba* associated with bromeliads in Rio Grande do Sul, Brazil, and the necessity for further studies to determine the environmental distribution and the role of these species.

1. Introduction

Free-living amoebae (FLA) of *Acanthamoeba* genus are one of the most abundant and widely distributed protozoa in the environment (Caumo and Rott, 2011; Siddiqui and Khan, 2012). They are cosmopolite and were isolated from soil, saltwater, freshwater and air samples (Rodriguez-Zaragoza and Magana-Becerra, 1997; Schuster and Visvesvara, 2004; Siddiqui and Khan, 2012). FLA are able to resist to extreme conditions such as extended time of desiccation, high/low temperature, pH and radiation (Alves Dde et al., 2012; Schuster and Visvesvara, 2004; Thomas et al., 2008). Many species of *Acanthamoeba* are causative agents of infections and disease in humans and animals (Winck et al., 2011). They show an ecological importance due their ability to be a reservoir of bacteria such as *Bacillus cereus*, *Escherichia coli*, *Helicobacter pylori*, *Legionella*

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pneumophila, *Mycobacterium avium*, and other "amoeba-resistant microorganisms", like viruses and fungi like *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Blastomyces dermatitidis* (Greub et al., 2004; Greub and Raoult, 2004; Khan, 2006; Siddiqui and Khan, 2012). These microorganisms are taken into the amoebae by phagocytosis and replicate inside the cytoplasm. The FLA act as "Trojan horses," protecting the microorganisms from harsh environmental conditions. After that, microorganisms return to the environment with implications of parasite–parasite interactions, which may contribute to the evolution and successful transmission of microbial in the environment. However, the exact nature of symbiosis and the benefit they represent for the amoebae host are still unclear (Greub and Raoult, 2004; Siddiqui and Khan, 2012).

According to Siddiqui and Khan (2012), *Acanthamoeba* genus plays two major ecological roles in soil: nutrient recycling and formation of the structure of microbial community. In general, the *Acanthamoeba* may develop an important function in the regulation of populations, contributing to the behavior of the ecosystems.

The plant surfaces, usually named phylloplane or phyllosphere are colonized by a large number of microorganisms (Slavikova et al., 2007). It is considered an important habitat for a variety of microorganisms (Andrews and Harris, 2000; Inacio et al., 2005). Leaf surfaces are colonized by members of many saprophytic microorganisms. The leaves are exposed to oscillation of temperature and relative humidity, which may represent an impact on the population of microorganisms. Large fluxes of ultraviolet light (UV) radiation are also another challenge in the leaf surface of which the microorganisms need to adapt (Lindow and Brandl, 2003).

Despite its large distribution in nature and its ecological importance, this study is the first report of FLA presence in the phylloplane of bromeliads. The aim of this study was to investigate the presence of FLA of *Acanthamoeba* genus in phylloplane of bromeliads in Southern Brazil, using molecular identification techniques and tolerance assays. The monitored *Acanthamoeba* community and the relationship that occur in the phylloplane are essential to the knowledge of ecological functions in nature, which need to be clarified.

2. Material and methods

2.1. Sample collection

Acanthamoeba were isolated from 10 samples of such bromeliads leaves: *Aechmea recurvata* ($n = 2$), *Vriesea gigantea* ($n = 2$) and *Vriesea friburgensis* ($n = 6$) in September of 2011 in Itapuã Park area, in South of Brazil (approx. coordinates: 30°22′S/51°04′W).

2.2. Isolation, axenization and cloning of FLA

Swabs were passed on leaves and placed inside sterile tubes containing 50 ml of sterile distilled water. They were gently shaken, squeezed and discarded. The collected material was maintained for 2 h for sedimentation process. After this time, material was centrifuged (250*g*, 10 min) and the supernatant was discarded. The sediment was resuspended in 0.5 ml of Page's saline. The suspension (100 μl) was used as inoculum on the 82-mm wide plate of 1.5% non-nutrient agar (NNA) containing an overlayer of an *Escherichia coli* (ATCC25922) suspension that had been heat inactivated (for 2 h at 56 °C). Plates were sealed with Parafilm® (SPI Supplies, West Chester, PA) and incubated at 30 °C for up to 15 days. Each plate was examined daily under a light microscope (at 100×) to check the presence of FLA. All positive samples were cloned by dilution method, where one microorganism is seeded in each isolate.

2.3. Molecular Identification of Acanthamoeba Isolates

Total DNA of each FLA-positive culture (containing 10^6 trophozoites) was extracted, as described by Salah and Iciar (Salah, 1997). The primers JDP1 and JDP2 were used to amplify the ASA.S1 region of the gene (Rns) coding for the amoeba's nuclear small-subunit ribosomal RNA (Schroeder et al., 2001). The polymerase chain reaction (PCR) was performed as described by Booton et al. (2004). Briefly, the amplifications were carried out in a 25 μl volume containing 20–30 ng of DNA, 0.2 mM dNTPs (Invitrogen™), 0.4 μM of each oligonucleotide, reaction buffer (50 mM KCl_2, 10 mM Tris–HCl), 1.5 mM $MgCl_2$, and 1 U of Platinum® Taq DNA Polymerase (Invitrogen™). Amplification conditions were as follows: one initial cycle at 95 °C for 5 min; 40 cycles at 95 °C for 60 s, 65 °C for 60 s, 72 °C for 60 s; and a final extension cycle at 72 °C for 5 min., The amplification product was separated in 1% agarose gel, stained with 0.5 μM/ml ethidium bromide, and observed under UV-light transilluminator. The sequences of strains were obtained with Amersham MegaBACE 1000 automated sequencers.

In order to classify the *Acanthamoeba* isolates, nine sequences of *Acanthamoeba* were uploaded into the Basic Local Alignment Search Tool (BLAST) program of the US National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) to search for

Table 1
Genotypic and physiological characterization of *Acanthamoeba* spp. isolates from phylloplane of bromeliads in Southern Brazil.

Isolate	Bromeliad/Source	GenBank acession	Genotype	GenBank acession reference sequences	Identity (%)	Tolerance assays[a]			
						Osmotolerance (M mannitol)		Thermotolerance (°C)	
						0.5	1.0	37	42
BRO2	*Vriesea friburgensis*	JX683392	T16	JQ408998	99%	+	–	+	–
BRO3	*Vriesea friburgensis*	JX683393	T2/T6	AY026244	99%	+	–	+	–
BRO4	*Vriesea friburgensis*	JX683394	T4	GU596997	99%	+	–	+	–
BRO5	*Vriesea friburgensis*	JX683395	T2/T6	AY026244	99%	+	–	+	–
BRO6	*Vriesea friburgensis*	JX683396	T2/T6	AY026244	99%	–	–	+	–
BRO7	*Aechmea recurvata*	JX683397	T2/T6	AY026244	99%	–	–	+	–
BRO8	*Vriesea friburgensis*	JX683398	T4	JQ418515	99%	+	–	+	–
BRO9	*Aechmea recurvata*	JX683399	T4	AY026243	98%	+	–	+	–
BRO10	*Vriesea gigantea*	JX683400	T2/T6	AY026244	98%	+	–	+	–
Neff – ATCC 30010[b]	Soil	K00471	T4	–	–	+	–	+	–
T4 – ATCC 50492[c]	Human cornea/ keratitis	U07401	T4	–	–	+	+	+	+

[a] (+) viability or growth (–) absence of growth.
[b] Environmental strain of *A. castellanii*.
[c] Clinical strain of *A. castellanii*.

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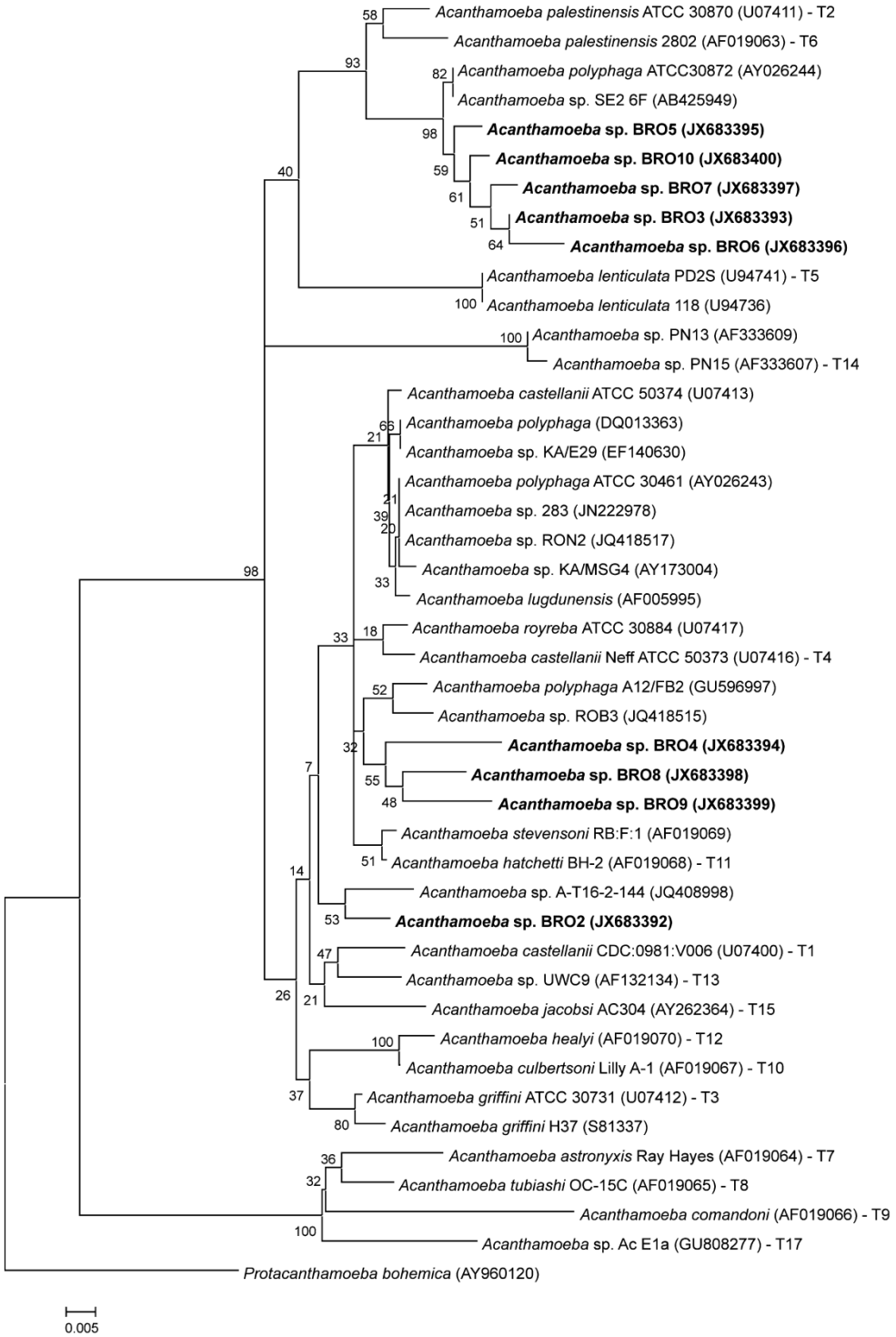

Fig. 1. Neighbour-joining 18S rDNA tree of the genus *Acanthamoeba* including all the genotypes and the bromeliads isolates. Bootstrap values (1000 replications) are shown above and below the nodes. GenBank accession numbers are in parentheses. Upper T after GenBank indicates type strain for the species. T1–T17 recognized *Acanthamoeba* genotypes. *Protacanthamoeba bohemica* (Acanthamoebida) was used as outgroup. Bar, 0.005 substitutions per nucleotide position.

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the most similar reference sequences. Alignments and phylogenetic trees were constructed with MEGA 5 (Tamura et al., 2011), using the neighbor joining method, with the bootstrap based on 1000 random samplings.

2.4. GenBank accession numbers

The 18S rRNA gene sequences of the nine *Acanthamoeba* isolates determined in this study were deposited in the GenBank database under the accession numbers JX683392 to JX683400.

2.5. Tolerance assays

Tolerance assays were performed in triplicate and the environmental (ATCC 30010 – Neff) and clinical (ATCC 50492 – T4) strains of *Acanthamoeba castellanii* were used as reference examples of nonpathogenic and pathogenic isolates, respectively.

2.5.1. Osmotolerance

Acanthamoeba trophozoites of each isolate were transferred (at 10^3 trophozoites/plate) to the center of fresh plates of 1.5% non-nutrient agar without mannitol (as a control) or containing 0.5 M or 1.0 M of mannitol each, with the usual overlayer of *Escherichia coli* suspension. The plates were incubated at 30 °C for 10 days and the growth was evaluated. The number of trophozoites or cysts observed (about 20 mm from the center of each plate) in five microscope fields at 100 × were counted, and the presence of growth and no growth being scored as positive (+) and negative (−), respectively (Caumo et al., 2009).

2.5.2. Thermotolerance

For temperature tolerance, plates were inoculated as described above and incubated at 30 °C (as a control), 37 °C, or 42 °C during 10 days. The result was evaluated as described above.

3. Results and discussion

All 10 samples of bromeliads leaves were positive for FLA and the 9 amoebae detected were identified, by morphology, as belonging to the genus *Acanthamoeba* (Caumo et al., 2009). As part of a more comprehensive study examining the genotype of *Acanthamoeba* spp. isolates obtained from bromeliads leaves, we identified *Acanthamoeba* genotypes by using 18S rDNA sequencing. Amplification of genus-specific amplimers ASA.S1 with primers JDP1 and JDP2 was successful in 9 isolates tested.

BLAST homology search showed all sequences were 98–99% identical to *Acanthamoeba* spp. deposited in GenBank (Table 1). The nine 18S rRNA sequences from the *Acanthamoeba* isolates were aligned with *Acanthamoeba* sequences retrieved from GenBank and phylogenetic analysis of the sequences showed that 5 isolates were clustered into genotype T2/T6. Three isolates were clustered into genotype T4, the same as pathogenic species such as *A. castellanii* strain Neff, and one into genotype T16 (Fig. 1).

The isolates BRO3, BRO5, BRO6, BRO7 and BRO10 are categorized into T2/T6 genotype revealed close identity (98–99%) to the reference strain *A. polyphaga* ATCC 30872, an environmental isolate initially classified in genotype T4 (Alves et al., 2000). However, Corsaro and Venditti (2010) proposed reclassify it as a distinct clade within the T2/T6 lineage, presenting genetic and phylogenetic data supporting that *Acanthamoeba polyphaga* ATCC 30872 showed similarities with both T2 and T6 genotypes.

The isolates BRO4, BRO8 and BRO9 were classified as T4 genotype. Many previous studies similarly reported that T4 is the most prevalent genotype among both clinical specimens and environment samples (Fuerst et al., 2003; Maghsood et al., 2005; Nuprasert et al., 2010). The presence of T4 isolates in our samples probably reflects their better adaptation to limited growth condition relative to isolates from other genotypes (Table 1).

The isolate BRO2 is categorized into the T16 genotype. The T16 genotype is less prevalent in the environment than T2/T6 and T4 and it was classified from an *Acanthamoeba* recovered from an environmental sample and from various unidentified *Acanthamoeba* sequences retrieved from GenBank (Corsaro and Venditti, 2010).

The results of the tolerance assays are summarized in Table 1. All isolates were able to grow at 37 °C incubation, no growth was observed at 42 °C and the isolates BRO2, BRO3, BRO4, BRO5, BRO8, BRO9 and BRO10 showed osmotolerance to 0.5 M D-mannitol but not to 1 M. The isolates BRO6 and BRO7 did not show osmotolerance to 0.5 M. The *in vitro* growth of an *Acanthamoeba* isolate under relatively high osmotic stress or at a relatively high temperature can be related to virulence, since virulence is at least partially, associated with an isolate's capacity to adapt and remain viable in the tissues of a mammalian host (Khan, 2006; Khan and Tareen, 2003). However, thermotolerance and osmotolerance assays are not necessarily synonymous of pathogenicity or virulence and *in vivo* assays are needed to characterize the pathogenic potential of FLA environmental isolates, because they may not be expressing its virulence factors, such as the clinical isolate that showed tolerance to all conditions tested. Other studies have demonstrated that a higher environmental temperature would promote the growth of thermotolerant *Acanthamoeba*. It is believed that such isolates could have evolved through natural selection to adapt to the heat stress in their niche (Rivera et al., 1993). The site of *Acanthamoeba* isolation in the present study, i.e. bromeliads leaves, are either at low and high temperature. The leaves are exposed to rapidly fluctuating temperature and the temperature is one of the most prominent features to which microorganisms have presumably had to adapt (Lindow and Brandl, 2003).

Despite the ubiquity of *Acanthamoeba* to be known, the present is the first study demonstrating the presence of *Acanthamoeba* spp. associated with bromeliads. *Acanthamoeba* spp. were detected in 90% of the bromeliads samples, confirming the wide-spread distribution of these FLA in the environment. The thermo and osmotolerance assays are insufficient condition for the determination of pathogenicity of the *Acanthamoeba* isolates collected and other tests will be conducted in the future to determine the pathogenic potential of the isolates. Also, since the environmental *Acanthamoeba* spp. feed on surrounding fungal, virus and bacteria free-living, they could be host for certain endosymbionts microorganisms. However, it is important to understand the interactions between *Acanthamoeba* spp. and the surrounding microorganisms and their ecological role in phylloplane needs to be investigated.

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