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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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uch 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.

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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*.

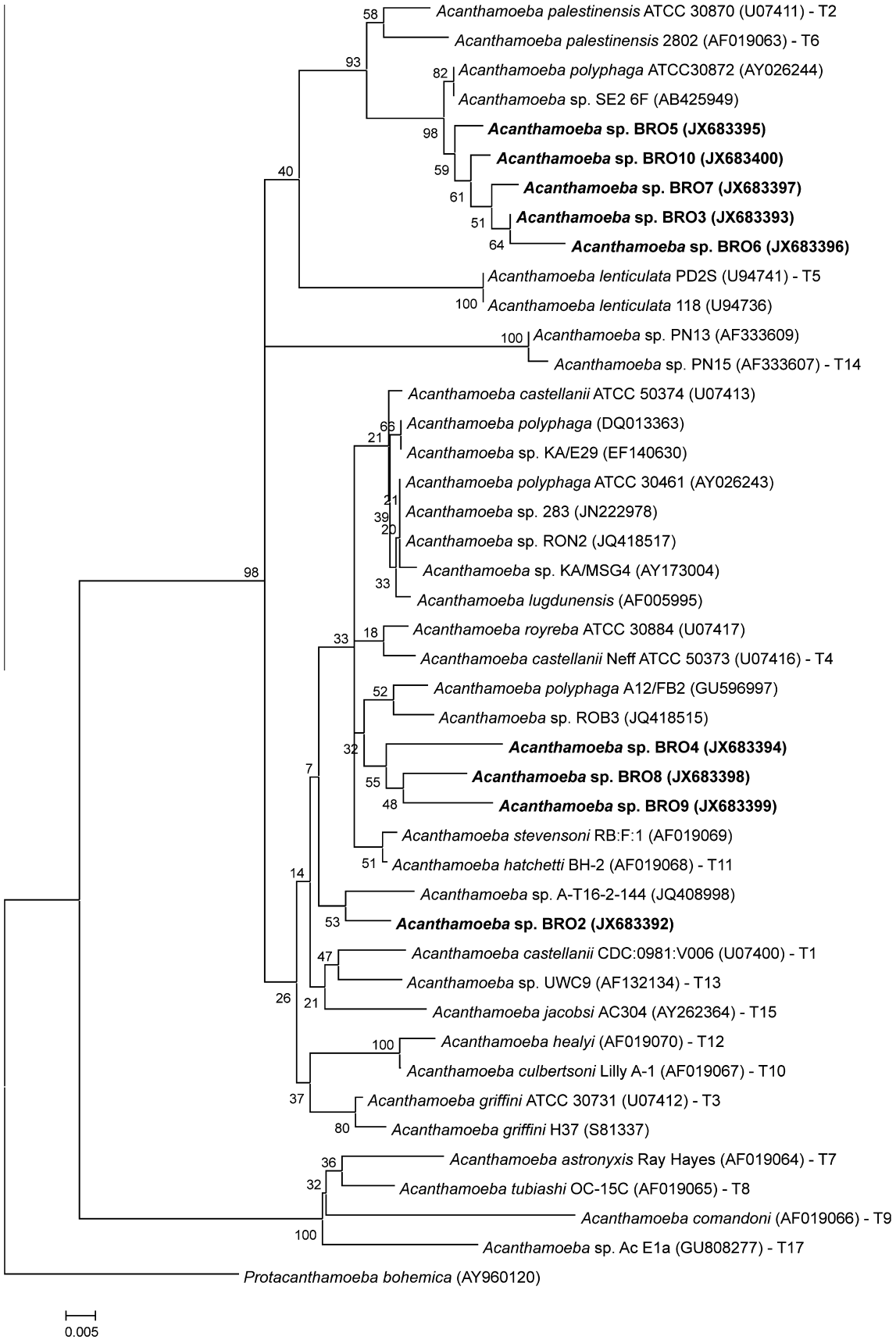

Fig. 1. Neighbour-joining 18S rDNA tree of the genus *Acanthamoeba* including all the genotypes and the bromeliads isolates. Bootstrap values (1000 replications) are shown above and below the nodes. GenBank accession numbers are in parentheses. Upper T after GenBank indicates type strain for the species.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

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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