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BRIEF COMMUNICATION

IDENTIFICATION OF *Pseudomonas* spp. AS AMOEBA-RESISTANT MICROORGANISMS IN ISOLATES OF *Acanthamoeba*

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SUMMARY

Acanthamoeba is a ''Trojan horse'' of the microbial world. The aim of this study was to identify the presence of *Pseudomonas* as an amoeba-resistant microorganism in 12 isolates of *Acanthamoeba.* All isolates showed the genus *Pseudomonas* spp. as amoeba-resistant microorganisms. Thus, one can see that the *Acanthamoeba* isolates studied are hosts of *Pseudomonas*.

KEYWORDS: *Acanthamoeba*; *Pseudomonas*; Amoeba-resistant microorganism.

Acanthamoeba is an opportunistic human pathogen that is ubiquitously distributed in the environment[13]. It is a causative agent of cutaneous lesions, sinus infections, vision threatening keratitis and rare but fatal encephalitis, known as granulomatous amoebic encephalitis. In addition, it has the ability to act as a host/reservoir for microbial pathogens[10,16].

Free-living amoebae feed by phagocytosis mainly on bacteria, fungi, and algae, and digestion occurs within phagolysosomes. Some microorganisms have evolved and have become resistant to predation by protists, since they are not internalized or are able to survive, grow, and exit free-living amoebae after internalization. *Acanthamoeba* is shown to be host/reservoir for numerous bacteria, including the genus *Pseudomonas* spp., among other bacterial pathogens[13].

Pseudomonas spp. are highly adaptable bacteria that can colonize various environmental niches, including soil and marine habitats, plants and animals. *Pseudomonas* spp. are also opportunistic human pathogens, causing infection of the eyes, ears, skin, urethra and respiratory tract in cystic fibrosis (CF) in burned patients, as well as other immunocompromised individuals[15].

In nature, free-living amoebae of the genus *Acanthamoeba* feed by *Pseudomonas* spp., which are widely distributed in the environment. Their encounter may be facilitated through better adherence of *Pseudomonas* spp. (than *E. coli*) to *Acanthamoeba* [2]. However, some *Pseudomonas* spp. have evolved to become resistant to predation by amoebae, as demonstrated by the isolation of *Acanthamoeba* naturally infected with *P. aeruginosa*[6,13]. Hence, free-living amoebae might also play a role as a reservoir for some amoeba-resistant strains of *Pseudomonas*, similar to what was shown for *Legionella* spp.[6]. This is important, given the role of *Pseudomonas aeruginosa* as a causative agent of pneumonia[5]. *Acanthamoeba* has been isolated from contact lens care systems contaminated with Gram-negative bacteria, including *Pseudomonas aeruginosa*[6].

Many studies have evaluated the interaction between *Acanthamoeba* spp. and *Pseudomonas* spp., as well as investigated the presence of these bacterial genera as amoeba-resistant bacteria[3,8,11].

In this study, the conventional technique of Polymerase Chain Reaction (PCR) was used, in order to identify the presence of the genus *Pseudomonas* spp. as amoeba-resistant microorganisms in isolates of *Acanthamoeba*.

A total of 12 environmental samples existing in the laboratory were used in this study: seven isolates from air-conditioning units identified as *Acanthamoeba* A2, A3, A4, A5, A7, A8 and A10, and five isolates from contact lens cases, *Acanthamoeba* A1, A6, A9, A11 and A12. The isolates were cultured in PYG media at 30 ºC (2% protease peptone, 0.2% yeast extract, and 1.5% glucose) supplemented with penicillin and streptomycin (Life Technologies). The total DNA in the sample was extracted, as described by ALJANABI & MARTINEZ[1]. The fresh culture containing 10^6 trophozoites was homogenized in 400 µL of sterile salt homogenizing buffer (0.4 M NaCl 10 mM Tris–HCl pH 8.0 and 2 mM EDTA pH 8.0), then, 40 µL of 20% SDS (2% final concentration) and 8 µL of 20 mg/mL protenase K (400 µg/mL final concentration) were added and mixed well. The samples were incubated at 65 ºC for, at

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least, one h, after which 300 µL of 6 M NaCl (NaCl saturated H_2O) was added to each sample. Samples were vortexed for 30s at maximum speed, and tubes spun down for 30 min at 10,000 x *g*. The supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample and samples were incubated at -20 ºC for one h. Samples were then centrifuged for 20 min, at 4 ºC and at 10,000 x *g*. The pellet was washed with 70% ethanol, dried and finally resuspended in 100 µL sterile dH_2O.

After extraction, the isolates were screened for the presence of bacterial endosymbiont - Bacteria domain - through the 16S rDNA gene amplified by PCR, using primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') that amplify 1500 bp in size, described by WEISBURG *et al.*[17], under the following conditions: five min at 94 °C, followed by 35 cycles of one min at 94 °C, one min at 55 °C and one min at 72 °C.

The identification of the presence of *Pseudomonas* genus DNA occurred using the primers described by SPILKER *et al.*[14] PA-GS-F (5'- GACGGGTGAGTAATGCCTA-3') and PA-GS-R (5'-CACTGGTGTTCCTTCCTATA-3') that amplifies 618 pb in size. Amplification was performed in a total volume of 25 µL containing 30 ng DNA, 10 pmol each primer, 5 pmol dNTP, reaction buffer (50 mM KCl_2, 10 mM Tris–HCl), 1.5 mM $MgCl_2$, and 1 U of Platinum Taq DNA Polymerase (InvitrogenTM). The amplification reaction was carried out in a PTC-150 Minicycler MJ Research thermocycler, under the following conditions: five min at 94 °C, followed by 35 cycles of one min at 94 °C, one min at 58 °C and one min at 72 °C.

The amplification product was separated in 1% agarose gel, stained with 0.5 µM/mL ethidium bromide and observed under a UV-light transilluminator. PCR products were purified using a QIAquick purification kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions, and resolved with a MegaBace 1000 automated sequencer. Analysis of the DNA sequences was performed with the Chromas Lite program and compared to those present in GenBank (http://blast.ncbi.nlm.nih.gov/).

In the present study, all isolates of *Acanthamoeba* showed internalized bacteria when primers are used to amplify the Bacteria domain and all isolates showed the genus *Pseudomonas* spp. as amoeba-resistant microorganisms (Fig. 1). A total of six PCR products (Ap1 to Ap6) were sent for sequencing (Table 1) and all were confirmed as *Pseudomonas* spp.

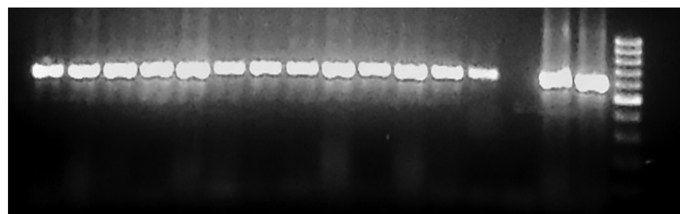

Fig. 1 - Samples of *Acanthamoeba* A1, A6, A9, A11 and A12 isolated from contact lens cases, and A2, A3, A4, A5, A7, A8, and A10 isolated from air conditioning units. Positive control (PC) strain of *Pseudomonas aeruginosa* ATCC 278532.

CALVO *et al.*[3] analyzed *Acanthamoeba* spp. originated from natural and anthropogenic environments and recorded the presence of *Pseudomonas* spp. as amoeba-resistant microorganisms in 26.1% of the isolates. GARCIA *et al.* (4) evaluated isolates from water coming from reservoirs and obtained 32.6% positive for *Pseudomonas* spp. In a study on clinical isolates of *Acanthamoeba* spp., IOVIENO *et al.*[8] observed that *Pseudomonas* spp. were present as amoeba-resistant microorganisms in 59% of the isolates studied.

Pseudomonas spp. have also been reported to be involved in keratitis and fatal pneumonia[7], among other diseases. Their presence may have a great impact on immune-suppressed individuals, since around 96% of the *Pseudomonas* spp. isolated from hot tubs and indoor swimming pools in a surveillance study display antimicrobial resistance[9]. Therefore, their prevalence in the environment, not only in recreational water but as part of biofilms in systems of distribution of drinking water, as well as their relevance in human pathogenicity led researchers to seek for its occurrence in amoeba hosts[3].

Table 1

Percentage of similarity and access number compared to GenBank sequences of identified bacteria in this study

Acanthamoeba	Fragment from the gel (GenBank accession)	Similarity BLAST	Access GenBank (number for access)
A1	Ap1 (KF160336)	98%	*Pseudomonas* sp. c145(2012) 16S ribosomal RNA gene, partial sequence (JQ781629.1)
A3	Ap2 (KF160337)	96%	Uncultured *Pseudomonas* sp. clone 3F10 16S ribosomal RNA gene, partial sequence (HM438578.1)
A4	Ap3 (KF160338)	99%	*Pseudomonas* sp. CJ-S-R2A3 16S ribosomal RNA gene, partial sequence (HM584286.1)
A6	Ap4 (KF160339)	99%	*Pseudomonas* sp. c145(2012) 16S ribosomal RNA gene, partial sequence (JQ781629.1)
A10	Ap5 (KF160340)	99%	*Pseudomonas* fluorescens strain C-D-TSA4 16S ribosomal RNA gene, partial sequence (HM755599.1)
A12	Ap6 (KF160341)	97%	*Pseudomonas* sp. c145(2012) 16S ribosomal RNA gene, partial sequence (JQ781629.1)

The possible role of *Acanthamoeba* as an evolutionary precursor of pathogenicity in microbial pathogens has been suggested[12]. Bacteria or other microbial endosymbiont may also enhance the pathogenicity of *Acanthamoeba*[12]. However, the results have been inconclusive. There are a few reports suggesting that amoeba-resistant microorganisms enhance the virulence of *Acanthamoeba*[6].

In addition to the bacteria identified in this work, the presence of other pathogenic amoeba-resistant microorganisms in the water samples tested cannot be discarded. *Acanthamoeba* spp. are also potential reservoirs of *Mycobacterium* spp.[3] and *Legionella* spp., among others microorganisms[3].

RESUMO

Identificação de *Pseudomonas* spp. como microrganismo resistente a ameba em isolados de *Acanthamoeba*

Acanthamoeba é um ‘’ Cavalo de Tróia’’ do mundo microbiano. Este estudo teve como objetivo identificar a presença de *Pseudomonas* como microrganismo resistente a ameba em 12 isolados de *Acanthamoeba*. Todos os isolados apresentaram o gênero *Pseudomonas* spp. como um microrganismo resistente a ameba. Assim, podemos ver que os isolados de *Acanthamoeba* estudados são hospedeiros de *Pseudomonas.*

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