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STAGGEMEIER, RODRIGO; ARANTES, THALITA; CAUMO, KARIN S.; ROTT,
MARILISE B.; SPILKI, FERNANDO R.

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Detection and quantification of human adenovirus genomes in *Acanthamoeba* isolated from swimming pools

RODRIGO STAGGEMEIER¹, THALITA ARANTES², KARIN S. CAUMO³,
MARILISE B. ROTT³ and FERNANDO R. SPILKI¹

¹Laboratório de Microbiologia Molecular, Universidade Feevale, RS-239, 2755, 96652-000 Novo Hamburgo, RS, Brasil

²Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul/UFRGS, Rua Sarmento Leite, 500, 90050-170 Porto Alegre, RS, Brasil

³Laboratório de Parasitologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul/UFRGS, Rua Sarmento Leite, 500, 90050-170 Porto Alegre, RS, Brasil

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ABSTRACT

Acanthamoeba is the most common free-living environmental amoeba, it may serve as an important vehicle for various microorganisms living in the same environment, such as viruses, being pathogenic to humans. This study aimed to detect and quantify human adenoviruses (HAdV) in *Acanthamoebas* isolated from water samples collected from swimming pools in the city of Porto Alegre, Southern Brazil. Free-living amoebae of the genus *Acanthamoeba* were isolated from water samples, and isolates (n=16) were used to investigate the occurrence of HAdVs. HAdV detection was performed by quantitative real-time polymerase chain reaction (qPCR). HAdVs were detected in 62.5% (10/16) of *Acanthamoeba* isolates, ranging from 3.24×10^3 to 5.14×10^5 DNA copies per milliliter of isolate. HAdV viral loads found in this study are not negligible, especially because HAdV infections are associated with several human diseases, including gastroenteritis, respiratory distress, and ocular diseases. These findings reinforce the concept that *Acanthamoeba* may act as a reservoir and promote HAdV transmission through water.

Key words: *Acanthamoeba*, free-living amoebae, human adenovirus, qPCR, swimming pools.

INTRODUCTION

Contamination of water sources by viruses, bacteria, and protozoa may cause outbreaks of severe disease, and the burden of gastroenteritis is a major public health problem worldwide (Scheid and Schwarzenberger 2012). Human adenoviruses (HAdVs) have been associated with outbreaks of waterborne diseases, inducing gastroenteritis, respiratory infections, conjunctivitis, meningitis,

and encephalitis, among other diseases (Kocwa-Haluch 2001, Carter 2005). Most HAdV infections are clinically unapparent or associated with mild symptoms. However, serious and sometimes fatal infections may occur, especially among the young, elderly, and immunocompromised (Lorenzo-Morales et al. 2007). HAdVs belong to the family *Adenoviridae*, genus *Mastadenovirus*, which includes more than 60 serotypes, divided into seven species (A to G) (Matsushima et al. 2013). The virus particles are nonenveloped, ranging from 70

Correspondence to: Rodrigo Staggemeier
E-mail: rstaggeimer@gmail.com

to 100 nm in diameter. The viral genome is a linear, double-stranded non-segmented DNA (Shenk 1996). HAdVs are excreted in large amounts in human feces and can be found in sewage, raw water, and treated water (Carter 2005).

Acanthamoeba, a free-living protozoan, is ubiquitous in water bodies, being characterized by high resistance to adverse environmental conditions, being able to survive in water even after conventional physical-chemical treatment for human consumption and recreational use, as well as after sewage treatment (Schuster and Visvesvara 2004). *Acanthamoeba* is the most common free-living environmental amoebae and has a cosmopolitan distribution. It has been isolated from a wide variety of habitats, such as freshwater lakes and rivers (Ettinger et al. 2003, Lorenzo-Morales et al. 2006), swimming pools (Gianinazzi et al. 2009), drinking water systems (Bernander and Kalling 1998), hospital hot water systems (Rohr et al. 1998), groundwater, bottled mineral water, and seawater (Lorenzo-Morales et al. 2005a). Furthermore, it can be found in soil (Lorenzo-Morales et al. 2005b), contact lens cleaning solutions (Gianinazzi et al. 2009), and surgical material (Foronda 1996). In humans, this protozoan occurs in the gastrointestinal tract, respiratory tract, and central nervous system (Kodet et al. 1998). Amoebae are unicellular phagocytes that feed on microorganisms in their environment, especially inert particles larger than 0.5µm (Raoult and Boyer 2010). Phagocytic amoebae may harbor different bacteria, fungi, and viruses within the same cell. Free-living amoebae can be pathogenic to humans and have been associated with subclinical infections, skin ulcerations, corneal infections, sinusitis, pneumonitis, and meningoencephalitis (Schuster and Visvesvara 2004).

Recreational water is clearly an important source of exposure to *Acanthamoeba* (Lorenzo-Morales et al. 2005b). The presence of *Acanthamoeba* strains in swimming pools has been documented in several studies (Rivera et al. 1993, Tsvetkova et al. 2004, Gianinazzi et al. 2009). In Poland (Górník and Kuzna-Grygiel 2004) and Finland (Vesaluoma et al. 1995), *acanthamoebae* were detected in swimming pool water, and in Chile, several genera of amoebae were detected, including *Acanthamoeba* (Muñoz et al. 2003). In Brazil, Chaves et al. (1985) found *acanthamoebae* in swimming pools located in the city of Campo Grande, state of Mato Grosso do Sul, Central-Western Brazil. In the Lagoa dos Patos estuary, Southern Brazil, Sassi et al. (2010) detected the presence of *Acanthamoeba* in 91.7% (11/12) of the isolates obtained. Some authors report that these amoebae reach the pools through the water supply, by air or even by swimmers themselves (Muñoz et al. 2003). In the state of Paraná, Southern Brazil, Ruthes et al. (2004) diagnosed conjunctivitis caused by *Acanthamoeba* in four patients; one of them did not wear contact lenses but used to swim at the club weekly, which the authors suggested might have led to ocular infection with *Acanthamoeba*.

It is known that amoebae act as predators in the control of microbial communities, serving as an environmental reservoir of pathogens and a vehicle for phylogenetically diverse microorganisms while some of them replicate intracellularly (Barker and Brown 1994, Scheid 2014). *Acanthamoebae* have demonstrated the ability to serve as hosts for pathogenic bacteria, such as *Legionella* spp., *Mycobacterium avium*, *Listeria monocytogenes*, and *Escherichia coli* serotype O157, among others (Greub and Raoult 2004). Studies on the relationship between viruses and free-living amoebae emerged in the early 1980s, when Danes and Cerva (1981) demonstrated an association between echoviruses and amoebae. Mattana et al. (2006) reported the capacity of coxsackie B3 viruses to survive within

amoebae for a 6-month cycle; thus, human viral pathogens may be phagocytized by these protozoa. More recently, a number of giant viruses, such as *Mimivirus*, *Marsseillevirus* (Raoult and Boyer 2010), and *Pandoravirus* (Philippe et al. 2013, Scheid et al. 2008, 2014), have been discovered in *acanthamoebae*. Such findings suggest that these amoebae have the ability to serve both as a reservoir of and as a replication niche for selected viruses (Raoult and Boyer 2010). Some studies have reported on patients coinfecting with human pathogenic viruses and amoebae, including HAdV and *Acanthamoeba* (Gajdatsy et al. 2000). The first description of the relationship between HAdV and amoebae in nature was published by Lorenzo-Morales et al. (2007), in which HAdV were detected in 14.4% (34/236) of environmental isolates from amoebae, suggesting that amoebae may play a role as a reservoir of HAdVs. However, this study was conducted with conventional polymerase chain reaction (PCR), and no viral genome quantification was performed. The aim of the current study was to detect and quantify HAdVs in *Acanthamoeba* isolated from water samples collected from swimming pools in Southern Brazil.

MATERIALS AND METHODS

Acanthamoeba ISOLATES

Environmental *Acanthamoeba* isolates (n=16) were collected from swimming pools located in the city of Porto Alegre, Southern Brazil. *Acanthamoeba* isolation was performed according to Caumo et al. (2009).

VIRAL NUCLEIC ACID EXTRACTION

Viral nucleic acids (DNA, HAdV) were extracted using the RTP® DNA/RNA Virus Mini Kit (Invitex™, Berlin, Germany), according to the manufacturer's instructions. An initial volume of 400 µL of each concentrated water sample was used for extraction through a silica filter.

The resulting viral DNA was stored at -80°C until processing.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

HAdV molecular detection was performed by qPCR using the commercial kit Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies™ Corporation, Carlsbad, California, USA), according to the manufacturer's instructions. The qPCR reactions were optimized and carried out under the same conditions, and then used as controls for absolute quantification of viral DNA from prototype samples of HAdV-5. The qPCR was carried out in a thermal cycler equipped with the iQ5™ Real-Time PCR Detection System (Bio-Rad™ Laboratories, Hercules, California, USA). The following primers were used: VTB2-HAdVCf (5'-GAGACGTACTTCAGCCTGAAT-3') and VTB2-HAdVCr (5'-GATGAACCGCAGCGTCAA-3') (Wolf et al. 2010). In a 25 µL reaction volume, we used 12.5 µL of the mixture, 1 µL of each primer (20 pM), 5.5 µL of DNase-/RNase-free sterile water, and 5.0 µL of the nucleic acid extracted from each sample. All reactions included a denaturation cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 20s and combined annealing/extension at 55°C for 1 min. Fluorescence data were collected during the annealing/extension step. Then, a denaturation curve was obtained to confirm the specificity of amplification products (a melting step between 55 and 95°C). To generate standard curves, 10-fold serial dilutions of standard controls from 10⁻¹ to 10⁻⁵ were prepared, starting at 6.01x10⁷ genome copies (gc) per reaction (HAdV-5). All standard controls and samples were run in duplicate. A no template control (NTC) and a negative control were used in each run to confirm that there was no contamination in the assay. PCR product specificity was confirmed by high-resolution melting (HRM) analysis and electrophoresis.

RESULTS

HAdVs genomes were detected in 62.5% (10/16) of *Acanthamoeba* isolates, ranging from 3.24×10^3 to 5.14×10^5 DNA copies per milliliter of isolate. The results are summarized in Table I.

TABLE I
Detection of HAdV in Isolates of *Acanthamoeba*
(Genomecopies/mL).

Identification	Genome Copies /mL
Amoeba 81	4,89E+04
Amoeba PI4	1,28E+04
Amoeba PI8	Neg
Amoeba PI3	2,13E+04
Amoeba PA1	Neg
Amoeba 84	2,09E+05
Amoeba P7	6,70E+04
Amoeba PT5	Neg
Amoeba 95	2,46E+05
Amoeba PI2	Neg
Amoeba P05	3,24E+03
Amoeba 121	2,23E+05
Amoeba P7	Neg
Amoeba 123	8,53E+04
Amoeba PM6	Neg
Amoeba 78	5,14E+05
Neg: Negative	

DISCUSSION

HAdV and *Acanthamoeba* can be found in aquatic environments side by side (Lorenzo-Morales et al. 2007), and according to recent reports it seems very likely that both microorganisms may interact (Scheid and Schwarzenberger 2012). Because of the wide distribution and high resistance of HAdVs in aquatic environments, they have been recognized as potential indicators of human fecal contamination. Understanding their interaction with free-living microorganisms is, therefore, of great interest, especially because there are few studies on the interaction between viruses and protozoan in the literature (Battistini et al. 2013, Scheid and Schwarzenberger 2012). Free-living amoebae have

been reported to host a number of different viruses, such as giant viruses (Suhre 2005, La Scola et al. 2003), adenoviruses (Lorenzo-Morales et al. 2007), rotaviruses (Sepp et al. 1992, Benyahya et al. 1997), and coxsackieviruses (Mattana et al. 2006). In the present study, HAdVs were detected in 62.5% of *Acanthamoeba* isolates recovered from environmental samples (62.5%), a detection rate higher than that previously reported in Spain (14.4% of isolates). This may be an effect of the detection technique used in the present study, since qPCR is usually more sensitive than conventional PCR (Donaldson et al. 2002).

In this study, HAdV viral loads in *acanthamoebae* ranged from 10^4 to 10^5 gc/mL in 90% (9/10) of positive samples, while a single isolate had 3.24×10^3 gc/mL. To the best of our knowledge, no previous study has quantified HAdVs genomes in protozoa, thereby hindering a proper comparison. Quantification of HAdV in this study was expressed as the number of genome copies/mL of *Acanthamoeba* isolate (approximately 10^6 - 10^7 trophozoites per mL). Studies around the world show variations in the measurement of HAdVs in water, and this amount may be influenced by the presence of amoebae in water. In river water, HAdV concentrations of 10^2 - 10^4 gc/L (Choi and Jiang 2005), 10^1 - 10^4 gc/L (Albinana-Gimenez et al. 2009), and 10^3 - 10^5 gc/L (Haramoto et al. 2010) have been detected, reaching mean values as low as <1 gc/L (van Heerden et al. 2005), 5×10^2 gc/L (Wyn-Jones et al. 2011), and 10^1 - 10^2 gc/L (Hundes et al. 2006).

According to Scheid and Schwarzenberger (2012), free-living amoebae appear to be able to act as carriers or vectors of adenoviruses, since the internalized viruses are protected to a high degree against the adverse actions of various disinfection treatments by their host amoebae, thereby playing a certain role in the dispersal of adenoviruses. Nevertheless, those authors concluded that, although there was no proliferation of adenoviruses in

Acanthamoeba, the virus was able to maintain its infectivity. Their study was the first to prove the ability of free-living amoebae to phagocytize and ingest adenoviruses, and the use of fluorescence microscopy allowed the detection of such viruses within the cytoplasm of the *Acanthamoeba* trophozoites. Lorenzo-Morales et al. (2007) considered *Acanthamoeba* a potential reservoir of HAdVs in the aquatic environment, and Battistini et al. (2013) demonstrated the resistance of HAdV for 105 days internalized in amoebae. These protozoa may also serve as a replication niche or training fields for resistance to the microbicidal effects of phagocytic cells, including human macrophages, which represent the first line of defense against invading pathogens (Thomas and Greub 2010, Colson et al. 2013, Scheid 2014). Phagocytosis and macropinocytosis are the main internalization processes in amoebae, being similar to the mechanisms involved in mammalian cells (Ghigo 2010), and free-living amoebae can be simultaneously infected with viruses and other microorganisms, such as bacteria (Yutin and Koonin 2009). More importantly, amoebae play a significant role as a melting pot for genetic exchanges between viruses and bacteria hosted by amoebae, and, as a result, viruses and intracellular bacteria living in amoebae have a sympatric lifestyle and large chimeric genome repertoires (Raoult and Boyer 2010, Thomas and Greub 2010). The HAdVs found in the present study are more likely being internalized and maintained inside amoeba cells, since all the experiments were performed on *Acanthamoeba* monolayers at the third passage.

Assuming that amoebae are able to carry these pathogens and protect them from external agents, in addition to their association with the diseases mentioned above, the possibility of ocular *Acanthamoeba* and HAdV coinfection should be considered in non-bacterial and non-fungal cases (Gajdatsy et al. 2000, Lorenzo-Morales et al. 2007). This association should also be considered in cases

of pneumonia given the growing body of evidence showing that mimiviruses might cause pneumonia in humans and because respiratory HAdV can reach the respiratory system using the same vectors as those reported by giant viruses (Colson et al. 2013), being able to stand up to the microbicidal effects of human macrophages as they have the ability to resist phagocytic cells (Thomas and Greub 2010). The present findings reinforce the idea that *Acanthamoeba* may act as a reservoir and promote HAdV transmission through contaminated water.

RESUMO

Acanthamoeba é a mais comum ameba de vida livre no ambiente, pode atuar como um importante veículo para vários micro-organismos que vivem no mesmo ambiente, tais como vírus, e pode ser patogênica para humanos. Este estudo objetivou detectar e quantificar adenovírus humano (HAdV) em *Acanthamoebas* isoladas de amostras de águas coletadas de piscinas na cidade de Porto Alegre, no sul do Brasil. Amebas de vida livre do gênero *Acanthamoeba* foram isolados de amostras de água, e os isolados (n = 16) foram usados para investigar a ocorrência de HAdVs. Detecção de HAdV foi realizada usando reação em cadeia de polimerase quantitativa (qPCR) em tempo real. HAdVs foram detectados em 62.5% (10/16) dos isolados de *Acanthamoeba*, variando de 3.24×10^3 a 5.14×10^5 cópias de DNA por mililitro de isolado. As cargas virais de HAdV encontradas nesse estudo não são negligenciáveis, especialmente porque infecções por HAdV são associadas com diferentes doenças humanas, incluindo gastroenterites, doenças respiratórias e oculares. Este estudo reforça o conceito que *Acanthamoeba* poderia atuar com um reservatório e promover a transmissão de HAdV através da água.

Palavras-chave: *Acanthamoeba*, ameba de vida livre, adenovírus humano, qPCR, piscinas.

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