Investigating the role of *Acanthamoeba polyphaga* in protecting Human Adenovirus from water disinfection treatment

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Abstract

Human adenoviruses are responsible for a wide range of clinical infections and are present in aquatic environments, including river, seawater, drinking-water and sewage. Free-living amoebae (*Acanthamoeba*) in the same environments may internalize them and other microorganisms can act as a reservoir for the internalized viruses. In this study, we studied the interaction between *Acanthamoeba polyphaga* and Human Adenovirus type 5 (HAdV 5) to determine whether the amoeba played a role in protecting the internalized viruses from chemical disinfection. The efficacy of sodium hypochlorite disinfection against *A. polyphaga* and HAdV 5 either singly or in combination was assessed at three different concentrations. Individually, the amoeba were more resistant to chemical disinfection than HAdV 5 and remained alive after exposure to 5 mg/l of sodium hypochlorite. In contrast, HAdV 5 lost infectivity following exposure to 2.5 mg/l of sodium hypochlorite. When the amoeba and HAdV 5 were co-cultured, infectious virus was found in the cytoplasm of the amoeba at 5 mg/l disinfectant concentration. These findings suggest that the *A. polyphaga* is providing protection for the HAdV 5.

Keywords: *Acanthamoeba polyphaga*; Co-culture; Disinfection; Human adenovirus

Introduction

Human adenoviruses (HAdV) are the etiological agents for numerous symptomatic and asymptomatic infections affecting the respiratory tract (e.g., pharyngitis, pneumonia, and respiratory diseases), visual system (e.g., conjunctivitis and keratoconjunctivitis), and gastrointestinal tract. While these diseases often resolve spontaneously, fatal infections can occur among children, the elderly, and immunocompromised subjects (Mena and Gerba, 2008). HAdV from acute infections can be excreted in the feces, urine, and respiratory secretions over a prolonged time (World and Horwitz, 2007), and transmission occurs via contact with the eye, the fecal-oral route, or inhalation. Adenoviruses can be found throughout the environment as well in raw and treated sewage, surface, marine, and freshwater, in foods, and aerosolized (Carducci et al., 2013; Iannelli et al., 2014; Jiang, 2006; Petrinca et al., 2009; Rodríguez-Lázaro et al., 2012; Verani et al., 2014; Verheyen et al., 2009).

In conjunction with bacterial indicators, HAdV have been proposed as indicators of anthropogenic contamination of water due to wastewater, based on their prevalence

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in the population and the environment, their environmental resilience, and host specificity (Albinana-Gimenez et al., 2009; Bofill-Mas et al., 2006; Carducci et al., 2008; Silva et al., 2011). Previous studies have also demonstrated that HAdV can be internalized by free-living protozoa, including *Euplotes octocarinatus* (Battistini et al., 2013) and *Acanthamoeba* species (Lorenzo-Morales et al., 2007; Scheid and Schwarzenberger, 2012), and survive for a prolonged period inside these organisms. This raises the possibility that protozoa can act as reservoirs for viruses, protecting them from unfavorable environmental conditions and disinfection efforts (Scheid, 2015). This type of endocytobiosis allows *Acanthamoeba* species to serve as carriers for other pathogenic microorganisms including *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Staphilococcus aureus*, *Acinetobacter baumannii*, *Campylobacter jejuny*, and *Vibrio cholerae* (Barker and Brown, 1994; Cateau et al., 2014; Michel, 2006; Scheid et al., 2014; Thomas et al., 2010).

Acanthamoeba species are ubiquitous and can be found both in artificial habitats, such as contact lens and their storage cases (Marciano-Cabral and Cabral 2003; Scheid et al. 2008) and in natural environments such as air (Kingston and Warhurst, 1969; Rodriguez-Zaragoza et al., 1993), soil (Reyes-Batlle et al., 2014), and water. *Acanthamoeba* species are most prolific in aquatic environments as they proliferate in biofilms and feed on other microorganisms and organic matter (Trabelsi et al., 2012). The broad ecological niche that *Acanthamoeba* species occupy makes close proximity to humans inevitable, and they can become opportunistic pathogens causing diseases such as keratitis, or granulomatous amoebic encephalitis in immunocompromised patients, which is often fatal (Khan, 2005). Several *Acanthamoeba* species have been found in tap water samples, which has been attributed to the ability of the amoebae to infiltrate the water distribution system or to inadequate disinfection methods that cannot completely eliminate trophozoites and cysts (Bonilla-Lemus et al., 2010; Kilvington et al., 2004).

The cited above role for *Acanthamoeba* species as a reservoir for HAdV in the environment has been proposed following the discovery of viral DNA from 4 different serotypes of adenoviruses in 34 of 236 (14.4%) strains of *Acanthamoeba* species isolated from samples of tap water in the Canary Islands (Lorenzo-Morales et al., 2007). In addition, corneal co-infections caused by *Acanthamoeba* and pathogenic adenoviruses have also been reported (Gajdatsy et al., 2000), and are often underestimated as the clinical symptoms of amoebic keratitis and keratoconjunctivitis are very similar. Analysis of the interaction between HAdV and *Acanthamoeba castellanii* suggests that the amoebae do not digest HAdVs and instead act as viral carriers. Using electron microscopy, no morphological changes were observed after the virus entered the digestive vacuoles of the protozoa, suggesting that the virus retains its virulence (Scheid et al., 2010). In sum, these studies suggest that *Acanthamoeba* could contribute to the environmental spread of HAdV by protecting the viruses inside the trophozoites and cysts where the virus can survive following phagocytosis.

To date, relatively little is known about the infectivity of HAdV internalized by protozoa and their ability to survive and persist in aquatic environments following decontamination procedures. Therefore, the present study was undertaken to investigate the survival of HAdV internalized in *Acanthamoeba polyphaga*, and evaluate the ability of this protozoa to protect the virus from disinfection using chlorine at concentrations normally sufficient to inactivate aquatic viruses.

Material and Methods

The environmental resistance to disinfectants of the target microorganisms is increased by the protective action of biofilms, for *Acanthamoeba polyphaga*, and by the organic compounds for HAdV. To reduce the influence of these factors, the experiments were performed in controlled standard condition using sterile water suspension added with known concentration of disinfectant.

The experimental work was divided into three main phases: (1) a comparative study of the efficiency of internalization of HAdV 5 in *A. polyphaga* cells using virus freely suspended or adsorbed to cell debris; (2) an estimation of the doses of sodium hypochlorite sufficient to inactivate *A. polyphaga* cells and HAdV 5, individually; (3) an evaluation of the resistance of the internalized virus to disinfection with sodium hypochlorite.

***Acanthamoeba polyphaga* replication and quantification**: Cultures of *A. polyphaga* (CCAP 1501/18) obtained from the Culture Collection of Algae and Protozoa (UK) were grown in flasks of 25 cm^2 at a temperature of 24 °C with PYG (Peptone–Yeast extract–Glucose) medium for either 2 days or until a cellular concentration of 10^5 cells/ml was reached. The cells were counted in a Burker chamber following the addition of 1:1 Trypan Blue.

Virus replication and quantification: Human adenovirus type 5 – HadV 5 (VR-5) obtained from the American Type Culture Collection (ATCC CCL-2) was used for all experiments. This serotype is responsible for respiratory infections and replicates in He-La cells, causing a visible cytopathic effect. He-La cells were grown in 25 cm^2 flasks with RPMI and 2% fetal calf serum. Confluent cell cultures were inoculated with the viral suspension, incubated at 37 °C and observed daily for 5 days or until the appearance of a pronounced cytopathic effect (40% of the total). The infected cultures were subsequently submitted to 3 cycles of freeze-thawing to lyse the cells. According to previous studies (Alotaibi, 2011; Mattana et al., 2006), the resulting suspension was divided into two aliquots: the first aliquot was used directly for experiments with virus adsorbed to cell debris, while the second aliquot was centrifuged (4000 *g* for 10 min) and the supernatant was used for experiments with

free virus. The viral titer was determined using the method of Spearman–Karber and expressed as $TCID_{50}$ (Hamilton et al., 1977).

Immunofluorescence test

In order to visualize the localization of the viruses in the amoeboid cultures, we used the direct immunofluorescence (IF) method usually applied for identification of adenoviruses in infected tissue cultures (Scheid et al., 2010). Specifically, we used a commercial kit (REF 17-020, Argene, France) standardized for the detection of HAdV in respiratory secretions, without quantification of infected cells and previously utilized to study HAdV internalization in *Euplotes* (Battistini et al., 2013). According to protocol, 10 ml of the *A. polyphaga*-HAdV (containing 90 cells) was centrifuged at 175 × *g* for 3 min. The pellet was washed four times in 10 ml of sterile mineral water, suspended in 100 ml of mineral water and pipetted onto a 6-well slide, resulting in the distribution of 10 μl of cell suspension (containing about 9000 cells) in each well. Next, the slide was air-dried and fixed in cold acetone for 10 min. Virus-specific fluorescein-conjugated monoclonal murine antibodies (anti-Adenovirus GROUP FITC, Argene,) were applied to the sample wells, and the slides were incubated for 15 min at 37 °C in a humidified chamber. The slides were then washed, air dried, mounted with immunofluorescence mounting medium (Argene), and examined immediately under a fluorescence microscope using 25×, 40×, or 63× lenses (Leica DMR microscope, Milan, Italy) connected to Leica DC490 digital camera with image acquisition software (Leica IM1000, v.1.0). The presence of bright green fluorescence (adenovirus associated with the FITC-conjugated monoclonal antibody FITC) on a red background of cells (Evan's blue stain) was considered positive.

Real Time quantitative PCR (qPCR) for Human Adenovirus detection

HAdV 5 DNA was quantified using real time quantitative PCR (qPCR) according to a published protocol (Hernroth et al., 2002). Viral genome was extracted for the samples with QIAamp DNA mini Kit. All reactions, performed in triplicate for each sample, were conducted using Taqman PCR Master Mix (Applied Biosystems, USA). The genome copy numbers of HAdV 5 in tested nucleic acid extracts were extrapolated from the equation of the standard curve generated from the dilution series (range = 10^2–10^9) of known amounts of nucleic acids. Standard curves were constructed by cloning the entire hexon region of HAdV 41 into pBR322.18. Each dilution of standard DNA suspensions was run in triplicate. Standard precautions were applied in all assays, including separate areas for the different steps of the protocol. In each plate, both a non-template control (NTC) and a positive control (at known concentration of purified HAdV DNA) were assayed in separate tubes. The presence of enzymatic inhibitors was determined by testing both undiluted and 10-fold diluted extracted DNA for each sample. The extracted samples (10 μl) were analyzed in 96-well optical plates. The plates were read with an ABI 7300 sequence detector system (Applied Biosystems).

Test of internalization

Co-culture experiments with *A. polyphaga* and the two different viral suspensions (with and without cell debris) were performed in 24-well plates. In each well, a confluent monolayer of trophozoites (with an average concentration of 1.6×10^5 cells/ml) was seeded with 1 ml of sterile deionized water and 1 ml of either (1) virus + cell debris, (2) freely-suspended virus or (3) Dulbecco's Modified Eagle Medium (D-MEM) as control. The co-cultures were incubated for 24 h at 25 °C according to preliminary tests performed (multiplicity of infection – MOI = 0.25), and then subjected to immunofluorescence (Scheid and Schwarzenberger, 2012). The test was repeated three times and three slides (18 wells) were analyzed for each co-culture experiment.

Evaluation of resistance of *Acanthamoeba polyphaga* to sodium hypochlorite

The resistance of *A. polyphaga* to disinfection was evaluated using different concentrations of free chlorine (1, 2.5 and 5 mg/l) obtained by diluting sodium hypochlorite (7%, Carlo Erba, Milan, Italy) and measuring concentrations with the colorimetric method using diethyl-p-phenylenediamine (DPD). These doses were chosen because they are commonly used during water disinfection (WHO, 2011). The amoebae were gently removed by scraping the monolayer in the 24-well plate and subsequently introduced into 15 ml test tubes together with the growth medium. The suspension was then centrifuged at 200 × *g* for 8 min and the pellet was washed 3 times in sterile deionized water. Next, the amoebae were diluted in deionized water to obtain a concentration 2×10^5 cells/ml. Aliquots of 0.5 ml of this suspension were transferred into 4 test tubes, 3 of them containing 9.5 ml of different concentrations of NaOCl (1, 2.5 and 5 mg/l) and one containing 9.5 ml of water. Each test was carried out in triplicate.

Based on previously published amoeba disinfectants resistance experiments (Dupuya et al., 2014; Garcia et al., 2007) we performed the test over 24 h, whereupon the residual chlorine was neutralized with 2% sodium thiosulfate (0.1 M) and the cells were stained with Tripan blue (nonviable staining) and counted in a Burker chamber. Additionally, an aliquot of 2 ml from each test tube was inoculated in PYG and incubated at 24 °C for 3 days to assess protozoa vitality.

Evaluation of the resistance of HAdV 5 to sodium hypochlorite

The resistance of HAdV 5 was evaluated using the same free chlorine concentrations described above (*vide supra*) in the experiments with *A. polyphaga*. Aliquots of 0.5 ml viral suspension containing cell debris, with a titer of 3.9×10^4 $TCID_{50}$/ml were transferred into 4 test tubes, 3 of them containing 9.5 ml of different concentrations of NaOCl (1, 2.5 and 5 mg/l) and one containing 9.5 ml of water. Each test was carried out in triplicate. Based on previously published studies of HAdV resistance to chlorine disinfection (Baxter et al., 2007; Cromeans et al., 2010), we performed the tests over 24 h, whereupon the residual chlorine was neutralized with 2% sodium thiosulfate (0.1 M) and an aliquot of 1 ml of each sample was obtained to test viral infectivity by titration with Karber method.

Evaluation of the resistance of internalized virus to disinfection with sodium hypochlorite

In order to verify whether the protozoa's internalization of the virus protected it against disinfection, co-cultures of *A. polyphaga* and HAdV 5, prepared as previously described, were treated with the same free chlorine concentrations used in the above evaluation tests. Co-cultures of amoebae and 2 different viral suspensions (virus + cell debris and freely suspended virus) were incubated for 24 h, collected in 24-well plates and washed (three times) in sterile deionized water to remove residual PYG. For each test, a total of 8-well plates were prepared. Next, the pellet of infected amoebas was re-suspended in 2 ml of sterile water and this suspension was added in 3 test tubes containing 8 ml of NaOCl solutions at different concentrations and one tube containing 8 ml of sterile water (control). Each experiment was repeated three times for a total of 24 analyses. After 24 h of contact, the disinfectant was neutralized as previously described and the suspensions were centrifuged at 200×*g* for 8 min. The resulting pellets and supernatants were then analyzed separately. The pellet (containing amoebae) was re-suspended in 2 ml of sterile water. One ml of this suspension was obtained for immunofluorescence tests, in order to assess the localization of virus and 1 ml was obtained for cultural (800 µl) and biomolecular (200 µl) tests. To test for infectivity, the amoebae were lysed by 4 freeze–thaw cycles, the solution was then centrifuged at 300×*g* for 5 min in order to remove debris and the supernatant was seeded on cell cultures. For the Real Time PCR assays, the DNA was extracted from the suspension as above described. The supernatant was directly analyzed for infectivity by viral titration $TCID_{50}$ and submitted to DNA extraction followed by Real Time PCR for genome quantification.

Data analysis

All data were statistically evaluated using the statistical software "Graph Pad Prism". One-way analysis of variance (ANOVA) was used to compare the cell (and viral) concentrations according to free chlorine concentrations. Dunnett's test was used to compare each individual treatment with the control for the different disinfectant concentrations.

Results

Test of internalization

The titer of the viral suspension containing the cell debris was determined to be 7.5×10^4 $TCID_{50}$/ml, while that of freely suspended virus was found to be slightly lower: 4.2×10^4 $TCID_{50}$/ml. Despite this similarity, all co-cultures of *A. polyphaga* containing HAdV 5 + cell debris showed a clear immunofluorescence, while no fluorescence was detected in either the co-cultures with free virus or in the control cultures (Fig. 1).

Acanthamoeba polyphaga resistance to disinfectant

The amount of active Cl was measured using the colorimetric method DPD, both at the beginning of treatment and immediately following the 24-h contact with the amoebas. When compared with the beginning of treatment, the concentrations of residual Cl after disinfection were reduced by more than 50%.

Fig. 1. Results of immunofluorescence on *Acanthamoeba* suspensions alone or co-cultured with free HAdV (B) or HAdV + cell debris (C). Control (A).

Table 1. Positivity of the various tests on the different materials after disinfection. +: the test show positive results after disinfection (at any concentration). –: the test show negative results after disinfection (at any concentration). ND: Not Determined. Immunofluorescence is not applicable to supernatants.

	Immunofluorescence	Infectivity	PCR
Amoebas + HAdV + cell debris pellet	+	+	+
Amoebas + HAdV + cell debris supernatant	ND	–	+
Amoebas + free HAdV pellet	–	–	+
Amoebas + free HAdV supernatant	ND	–	+

One-way ANOVA revealed significant differences of chlorine concentrations between groups (control and the three concentrations of free chlorine) (R^2 = 0.5846 and p-value = 0.0121). Post-hoc analysis using Dunnett's test showed significant p-value ($p < 0.05$) only for the concentration of 5 mg/l compared to control. The log reduction remained low, increasing from 0.06 ± 0.07 for 1 mg/l of free chlorine, to 0.2 ± 0.17 for 2.5 mg/l free chlorine and to 0.29 ± 0.13 for 5 mg/l free chlorine. Cell viability was also confirmed by their growth in PYG.

HAdV 5 resistance to the disinfectant

Analysis of variance among free chlorine concentrations gave significant results for $p < 0.001$ (R^2 = 0.7414 and p-value = 0.0098) and post-hoc Dunnett's test showed significant p-values ($p < 0.01$) for each chlorine concentration compared to control. A total loss of infectivity was observed with 5 p.p.m. of free Chlorine, corresponding to a log reduction of 3.03 ± 0.00. The log reduction for the adenovirus titer was 2.89 ± 0.07 for 2.5 mg/l and 2.51 ± 0.31 for 1 mg/l.

Evaluation of the resistance of internalized virus to disinfection with sodium hypochlorite

The results of disinfection experiments are summarized in Table 1. Immunofluorescence and infectivity were found to be positive only for the pellet of co-cultures of amoebas with virus adsorbed to cell debris, while qPCR was positive for all samples.

Fig. 2 shows clear fluorescence in amoebas co-cultured with HAdV 5 + cell debris, while no fluorescence was visible in amoebas co-cultured with free virus.

For co-cultures with virus adsorbed on cell debris, no infectivity was observed in the supernatant (except in control samples not exposed to disinfectant), while evaluation of the amoeba pellet revealed the presence of viable virus after exposure at each of the free chlorine concentrations. Accordingly, the log reduction for the different disinfectant concentrations was similar: 2.73 ± 0.06 for 1 mg/l of chlorine, 2.71 ± 0.04 for 2.5 mg/l of chlorine and 2.71 ± 0.07 for 5 mg/l of chlorine.

Although qPCR results were uniformly positive, significant differences were not observed in the values for pellets from the free chlorine concentrations, while the values for supernatants showed a significant reduction according to these concentrations (Table 2).

Moreover, for the experiments carried out in co-cultures of amoebae with free virus, the qPCR values were lower for pellets and the reduction effect of chlorine was evident for supernatants, but not for the pellets (Table 3).

Discussion

Our study, performed in controlled laboratory condition, confirms that HAdV 5 can be better internalized in *A. polyphaga* if viruses are mainly adsorbed to cell

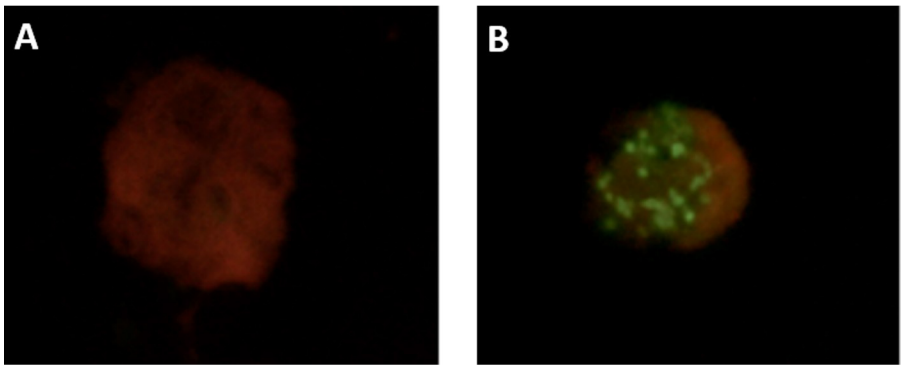

Fig. 2. Results of immunofluorescence on *Acanthamoeba* suspensions co-cultured with free HAdV 5 (A) or HAdV 5 + cell debris (B), and treated with free chlorine 5 mg/l.

Table 2. Average viral Log concentration and standard deviation (on three replicates) of genomic copies in supernatant and pellet of co-cultures with virus adsorbed to cell debris, after exposure to different free chlorine concentrations.

	Control	1 mg/l	2.5 mg/l	5 mg/l	ANOVA test (p)
Supernatant (Log GC)	4.74 ± 0.17	3.55 ± 0.24*	3.05 ± 0.12*	2.88 ± 0.53*	0.0003
Pellet (Log GC)	4.93 ± 0.79	4.69 ± 1.30	4.51 ± 0.05	4.63 ± 0.14	0.8993

* Dunnett's test resulted significant ($p < 0.05$).

Table 3. Average viral Log concentration and standard deviation (on three replicates) of genomic copies in supernatant and pellet of co-cultures with free HAdV, after exposure to different free chlorine concentrations.

	Control	1 mg/l	2.5 mg/l	5 mg/l	ANOVA test (p)
Supernatant (Log GC)	3.98 ± 0.76*	2.94 ± 0.22*	2.08 ± 0.11*	1.68 ± 0.13*	0.0005
Pellet (Log GC)	1.09 ± 0.70	0.97 ± 0.27	1.02 ± 0.25	0.89 ± 0.06	0.9287

* Dunnett's test resulted significant ($p < 0.05$).

debris, similar to previously published reports concerning *A. castellani* and coxsackievirus, demonstrating an absence of phagocytosis of freely suspended virus (Alotaibi, 2011; Mattana et al., 2006).

The ability of *A. polyphaga* to ingest and internalize other microorganisms depends on many factors, including size, movement and molecular structure (de Moraes and Alfieri, 2008). However, when found in natural solutions, viruses are normally adsorbed to organic compounds, suggesting that the likelihood of viral phagocytosis remains high. The internalization of viruses by protozoan species does not eliminate viral infectivity, but rather seems to increase its resistance in the environment. Mattana et al. (2006) reported that if trophozoites infected by coxsackievirus undergo encystment following exposure to adverse environmental conditions, the virus retains its infectivity even after a completed cycle of encystment–excystment. It is well known that adenoviruses have a high ability to survive in water, due to their double-stranded DNA and their ability to adsorb to other compounds. However, several studies have shown that adenoviruses are susceptible to inactivation by free chlorine (Shin et al., 2002; Thurston-Enriquez et al., 2003). Baxter et al. (2007) also reported that exposure of a purified viral suspension to 0.12 mg/l of free chlorine for 3 min resulted in a viral inactivation of 5-log.

In our experiments, we used a viral suspension containing cell debris, a chlorine concentration similar (or higher) to that normally found in swimming pools and a contact time of 24 h that reduced the final chlorine concentration by 50%. Our goal was to evaluate the resistance of adenovirus in a realistic situation such as that of a swimming pool, which represents the most common environmental context for infections caused by both *Acanthamoeba* and adenovirus (Al-Herrawy et al., 2014; Mena and Gerba, 2008; Rivera et al., 1983; Sinclair et al., 2009). We found that the resistance of HAdV 5 alone was elevated when compared to values from previous studies in which the virus was tested free (Baxter et al., 2007; Page et al., 2009), with a log reduction of 3 at 5 mg/l of free chlorine for 24 h. Conversely, *A. polyphaga* showed a higher chlorine resistance with a very low reduction under identical standard laboratory controlled conditions. Similar results were obtained for internalized virus.

The immunofluorescence tests and the comparison of $TCID_{50}$ data, for pellets and supernatants of co-cultures with both adsorbed and un-adsorbed virus demonstrated that the disinfectant killed only the viruses in suspension, while viruses internalized in amoebae (in pellet of co-cultures with adsorbed virus) were able to survive. With respect to our results using qPCR, the positivity of all samples tested may be explained by the detection of viral DNA belonging to non-infectious viruses (inactivated by disinfection), as previously described (Bertrand et al., 2012; Gassilloud et al., 2003; Simonet and Gantzer, 2006). However, comparing quantitative data between internalized and suspended virus a statistical significance difference was observed.

Conclusion

Our results confirm that *A. polyphaga* could represent an important protective reservoir for HAdV 5 in disinfected waters. These results, obtained in laboratory experiments, have direct relevance if reported to a variety of environmental contexts such as drinking water treatment plants, spas and swimming pools, where both HAdV and *A. polyphaga* are known to be important pathogens, often causing outbreaks or sporadic cases of ocular infections (Caumo and Rott, 2011; Magnet et al., 2012; Mena and Gerba, 2008). The role of this association in the pathogenesis of keratitis should be further evaluated in future studies through the analysis of clinical samples. Furthermore, we suggest that future studies should also evaluate:

- The role of *A. polyphaga* adsorbed to biofilm in the internalization of viruses.
- The duration and persistence of viral infectivity in the amoeboid cytoplasm.

- The presence of adenovirus internalized in *A. polyphaga* in environmental water samples

Acknowledgements

We wish to thank Dr Beatrice Casini (University of Pisa) for *A. polyphaga* strain supplied and assistance. We thank Fabrizio Erra for technical support with Immunofluorescence microscopy and the native English-speaking experts of "BioMed Proofreading" for the help in editing this article.

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