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Research Paper

Free-living amoebae (*Acanthamoeba* spp.): diagnosis by PCR method in different sources of water in Rabat, Morocco

Sakhi El Mahdi, Moumni Mostafa, Radid Horia, Arahou Mohamed and Fekhaoui Mohamed

ABSTRACT

Free-living amoebae are ubiquitous protozoa, frequently found in the aquatic environment. *Acanthamoeba* spp., in some conditions, causes amoebic keratitis. Our research project aimed at studying *in vivo Acanthamoeba* spp. that are possibly present in water destined for human consumption. Thus, we can evaluate the rate of water contamination by determining the critical areas of its presence. In total, 150 water samples were analysed from Rabat. All the samples were collected from five different sources: river, fountain water, seawater, public bath water and tap water. The samples were distributed over three seasons: spring, summer and autumn. The positive samples by culture method were confirmed by polymerase chain reaction (PCR) method. The obtained results by microscopic identification and PCR method showed a high percentage of the presence of *Acanthamoeba* spp. in water in Rabat. However, during the sampling period, we noticed a non-uniform division of the positive samples with a remarkably high rate during summer. Our study showed that water contamination by *Acanthamoeba* spp. in Rabat, Morocco is at high risk of having a negative impact on public health. It is necessary to do a follow-up and study the health impacts to better evaluate the risk associated with this contamination by *Acanthamoeba* spp.

Key words | *Acanthamoeba* spp., *Acanthamoeba* keratitis, conventional PCR, free-living amoebae

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INTRODUCTION

Free-living amoebae are unicells, eukaryotic and ubiquitous, which are present in the aquatic environment contrary to pathogenic amoebae (associated with a host). Most of the genera are not pathogenic, some others are opportunists: *Acanthamoeba* spp. can cause, in certain conditions, *Acanthamoeba* keratitis. This disease becomes more frequent when using contact lenses (more than 80% of the cases) (Cabral & Cabral 2003; Lorenzo-Morales *et al.* 2015). *Acanthamoeba*, *Naegleria* and *Vahlkampfia* are the main genera which represent the aquatic free-living amoebae (Kuiper *et al.* 2006; Thomas *et al.* 2006; Corsaro *et al.* 2007). Moreover, the amoebae population is regulated by the presence of nutriments, physicochemical factors, pH and annual temperature fluctuations (Kyle & Noblet 1986; Rohr *et al.* 1998; Pumidonming *et al.* 2010). The source of keratitis infection is highly related to the use of water contaminated by *Acanthamoeba* spp. In Morocco, studies associated with water contaminated by free-living amoebae have never been done and there are no related statistical data. Our research project suggests studying, *in vivo*, free-living amoebae (*Acanthamoeba* spp.) that are present in water destined for human consumption; thereby, we

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can assess the degree of contamination of these waters by determining the critical areas.

MATERIALS AND METHODS

During the period from April to November 2016, 150 water samples were collected from Bouregreg river, fountains, taps, public baths and ocean from the Rabat region, Morocco. The samples were collected weekly from different points, depending on the water category. Subsequently, 30 samples were divided according to the three seasons: spring, summer and autumn. The samples were collected using plastic bottles with a capacity of 2 L and these were examined by parasitological analysis in the General Department of Parasitology of the National Institute of Hygiene, Rabat, Morocco.

Culture method

The culture method used was non-nutritive agar with attenuated bacterial substrate (NNE) *Escherichia coli* ATCC 25922. The samples were filtered by a pumping aspiration system using a filter (3 μm pore size) of nitrocellulose. The volume of filtration according to the type of samples was: clean water box 1 L and raw water box 1 L 500 mL. The filter side of the filter was deposited on the non-nutrient agar surface. Petri dishes were incubated at two different temperatures (20 °C and 30 °C) for 15 days. Two different temperatures were used to increase the possibility of detecting other free-living amoebae in the water. Observation of the Petri dishes was done through an inverted microscope (this observation was made using magnification of 40 ×). Positive Petri dishes were analysed using the conventional polymerase chain reaction (PCR) method.

Genomic method

The cysts of the amoebae and their possible migration traces observed in the Petri dishes were suspended in 300 μL of the lysis buffer (0.1 M Tris-HCl, 0.1 M EDTA, 0.1 M NaCl, 2% SDS). Before DNA extraction, several steps were used to lyse the double cellulose wall of amoeba cysts, where five cycles of thermal shock were used, alternating with 1 minute of incubation at a temperature of 95 °C and 1 minute in a bath of liquid nitrogen, mechanical lysis by glass beads, and enzymatic digestion with proteinase K for 1 hour at 37 °C. Subsequently, DNA extractions were performed using a Bioline kit according to the supplier's recommendations (Fields 1996; Dykova *et al.* 2005; Fouque *et al.* 2012; Trabelsi *et al.* 2012). In the PCR reaction (25 μL) each sample was prepared as follows: DNA template (1 μL), forward primer (1.5 μL) (ACA-F (5′–3′): TGG CAG CGC GAG GAC TAG GG) reverse primer (1.5 μL) (ACA-R (3′–5′): ACC GCA CCG ATG GTG GTG TTT) (Leduc *et al.* 2012), *Taq* buffer and *Taq* DNA polymerase (12 μL), and pure water (9 μL). The amplification of DNA was performed by using a PCR thermal cycler. The programme included an initial denaturation at 95 °C for 5 min followed by 35 cycles; each cycle consisted of denaturation at 95 °C for 1 min, annealing at 63 °C for 1 min and extension at 72 °C for 1 min. The programme included a final extension step at 72 °C for 10 min. The validation process of the 18S rDNA gene was performed by electrophoresis on agar-agar (1%), and the amplification of the fragment was visualized by using ethidium bromide.

RESULTS

Of the total 150 samples from the five sampling areas in Rabat, Morocco, the microscopic presence of *Acanthamoeba* spp. cysts was observed in 20 samples (13.33%). By means of the PCR method, 18 samples were positively obtained (Table 1). However, the distribution of positive samples during the three seasons has revealed an abundance of *Acanthamoeba* spp. in the summer period (Table 2). Table 2 shows the percentage of contamination of the waters of Rabat by *Acanthamoeba* spp. during the three seasons.

Table 1 | Distribution of positive samples at the different sampling sites

Sites of samples	Sample number	Positive numbers	% of positivity
Sea	30	7	23%
Bouregreg river	30	11	36%
Fountains	30	0	0%
Public bath wáter	30	0	0%
Tap	30	0	0%
Total	150	20	13%

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Table 2 | Distribution of *Achanthamoeba* spp. in three seasons

Seasons	Sample number	Positive numbers
Spring	50	0
Summer	50	15
Autumn	50	3
Total	150	18

DISCUSSION

The results obtained during sampling of the water samples revealed the presence of a high percentage of *Acanthamoeba* spp. in the Rabat area (Table 1), which may be due to bacterial diversity (Greub & Raoult 2004; Coleman *et al.* 2009). Bouregreg river has the highest pollution rate, unlike the samples collected from seawater (36.6% vs. 26.66%), respectively. The presence *of Acanthamoeba* spp. can be due to several factors, such as wastes and excretion products of livestock activity near tributaries, the presence of wildlife in rivers, as well as discharges of untreated wastewater and industrial emissions, especially from agriculture through the Bouregreg river (Laouina *et al.* 2010), which makes it the ideal environment for bacterial growth and development, particularly free-living amoebae (Adekambi *et al.* 2006; Pickup *et al.* 2007). The low pollution rate of seawater is related to the salinity of the Atlantic Ocean (33.5–37.4). Researchers have focused less on the study of *Acanthamoeba* spp. in seawater. This high salinity generates a limiting factor for the development of *Acanthamoeba* spp. (Booton *et al.* 2004; Steinum *et al.* 2008). Even so, water exposure to the air and soil contamination helps *Acanthamoeba* live and spread (Trabelsi *et al.* 2012). In Iran, researchers have indicated that *Acanthamoeba* spp. is present in 73.53% of environmental water samples (Mohammadi Manesh *et al.* 2016). Forty-eight per cent of the water collected from 14 cities is contaminated by *Acanthamoeba* spp. (Bagheri *et al.* 2010). In Taiwan, in 2014, 39.5% of the waters were contaminated by free-living amoebae (Kao *et al.* 2014). The increase in summer contaminated samples shows the impact of temperature on the development of the amoeba population (Niederkorn *et al.* 1999; Douglas-Helders *et al.* 2001; McAllum *et al.* 2009).

CONCLUSIONS

The number of samples and the limited filtration method are the two notable factors about uncontaminated waters in this study. Water pollution in the Rabat region can be considered a potential source of infection for people, and the use of contaminated water to wash the eyes can put people at risk of contracting *Acanthamoeba* keratitis. Therefore, public education, as well as prevention measures regarding *Acanthamoeba* spp. seems to be necessary in Rabat.

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REFERENCES

Adekambi, T., Ben Salah, S., Khlif, M., Raoult, D. & Drancourt, M. 2006 Survival of environmental mycobacteria in *Acanthamoeba polyphaga*. *Appl. Environ. Microbiol.* **72**, 5974–5981.

Bagheri, H., Shafiei, R., Shafiei, F. & Sajjadi, S. 2010 Isolation of *Acanthamoeba* spp. from drinking waters in several hospitals of Iran. *Iran. J. Parasitol.* **5** (2), 19–25.

Booton, G. C., Rogerson, A., Bonilla, T. D., Seal, D. V., Kelly, D. J., Beattie, T. K., Tomlinson, A., Lares-Villa, F., Fuerst, P. A. & Byersa, T. J. 2004 Molecular and physiological evaluation of subtropical environmental isolates of *Acanthamoeba* spp., causal agent of *Acanthamoeba* keratitis. *J. Eukaryot. Microbiol.* **51**, 192–200.

Cabral, M. F. & Cabral, G. 2003 *Acanthamoeba* spp. as agents of disease in humans. *Clin. Microbiol Rev.* **16** (2), 273–307.

Coleman, D. C., O'Donnell, M. J., Shore, A. C. & Russel, R. J. 2009 Biofilm problems in dental unit water systems and its practical control. *J. Appl. Microbiol.* **106** (5), 1424–1437.

Corsaro, D., Thomas, V., Goy, G., Venditti, D., Radek, R. & Greub, G. 2007 *Candidatus* Rhabdochlamydia crassificans, an intracellular bacterial pathogen of the cockroach *Blatta orientalis* (Insecta: Blattodea). *Syst. Appl. Microbiol.* **30**, 221–228.

Douglas-Helders, M., Saksida, S., Raverty, S. & Nowak, B. F. 2001 Temperature as a risk factor for outbreaks of amoebic gill disease in farmed Atlantic salmon (*Salmo salar*). *Bull. Eur. Assoc. Fish Pathol.* **21**, 114–116.

Dykova, I., Pindova, Z., Fiala, I., Dvorakova, H. & Machackova, B. 2005 Fish-isolated strains of *Hartmannella vermiformis* page, 1967: morphology, phylogeny and molecular diagnosis of the species in tissue lesions. *Folia Parasitol.* **52**, 295–303.

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Fields, B. S. 1996 The molecular ecology of legionellae. *Trends Microbiol.* **4**, 286–290.

Fouque, E., Trouilhe, M. C., Thomas, V., Hartemann, P., Rodier, M. H. & Hechard, Y. 2012 Cellular, biochemical and molecular changes during encystment of free-living amoebae. *Eukaryot. Cell.* **11** (4), 382–387.

Greub, G. & Raoult, D. 2004 Microorganisms resistant to free-living amoebae. *Clin. Microbiol.* **17**, 413–433.

Kao, P. M., Hsu, B. M., Hsu, T. K., Lin, J. H., Chang, H. Y., Ji, W. T., Tzeng, K. J., Huang, S. W. & Huang, Y. L. 2014 Seasonal distribution of potentially pathogenic *Acanthamoeba* species from drinking water reservoirs in Taiwan. *Environ. Sci. Pollut. Res. Int.* **22**, 3766–3773.

Kuiper, M. W., Valster, R. M., Wullings, B. A., Boonstra, H., Smidt, H. & Van Der Kooij, D. 2006 Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water by using real-time PCR. *Appl. Environ. Microbiol. Am. Soc. Microbiol.* **72**, 5750–5756.

Kyle, D. E. & Noblet, G. P. 1986 Seasonal distribution of thermotolerant free-living amoebae. *J. Protozool.* **33**, 422–434.

Laouina, A., Aderghal, M., AI Karkouri, J., Chaker, M., Machmachi, I., Machouri, N. & & Sfa, M. 2010 Land use, runoff and land degradation: the case of the Sehoul sector, Atlantic region (Morocco). *Drought* **21** (4), 309–316.

Leduc, A., Gravel, S., Abikhzer, J., Roy, S. & Barbeau, J. 2012 Polymerase chain reaction detection of potentially pathogenic free-living amoebae in dental units. *Can. J. Microbiol.* **7**, 884–886.

Lorenzo-Morales, J., Khan, N. A. & Walochnik, J. 2015 An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite* **22** (10), 1–10.

McAllum, P., Bahar, I., Kaiserman, I., Srinivasan, S., Slomovic, A. & Rootman, D. 2009 Temporal and seasonal trends in *Acanthamoeba* keratitis. *Cornea* **28**, 7–10.

Mohammadi Manesh, R., Niyyati, M., Yousefi, H. A. & Eskandarian, A. A. 2016 Isolation of *Acanthamoeba* spp. from different water sources in Isfahan, central Iran, 2014. *J. Parasit. Dis.* **40** (4), 1483–1486.

Niederkorn, J. Y., Alizadeh, H., Leher, H. & McCulley, J. P. 1999 The pathogenesis of *Acanthamoeba* keratitis. *Microbes Infect.* **1**, 437–443.

Pickup, Z. L., Pickup, R. & Parry, J. D. 2007 Effects of bacterial prey species and their concentration on growth of the amoebae *Acanthamoeba castellanii* and *Hartmannella vermiformis*. *Appl. Environ. Microbiol.* **73**, 2631–2634.

Pumidonming, W., Koehsler, M. & Walochnik, J. 2010 *Acanthamoeba* strains show reduced temperature tolerance after long-term axenic culture. *Parasitol. Res.* **106**, 553–559.

Rohr, U., Weber, S., Michel, R., Selenka, F. & Wilhelm, M. 1998 Comparison of free-living amoebae in hot water systems of hospitals with isolates from moist sanitary areas by identifying genera and determining temperature tolerance. *Appl. Environ. Microbiol.* **64** (5), 1822–1824.

Steinum, T., Kvellestad, A., Rónneberg, L. B., Nilsen, H., Asheim, A., Fjell, K., Nygard, S. M. R., Olsen, A. B. & Dale, O. B. 2008 First cases of amoebic gill disease (AGD) in Norwegian seawater farmed Atlantic salmon, *Salmo salar* L., and phylogeny of the causative amoeba using 18S cDNA sequences. *J. Fish Dis.* **31**, 205–214.

Thomas, V., Herrera-Rimann, K., Blanc, D. S. & Greub, G. 2006 Biodiversity of amoebae and amoebae-resisting bacteria in a hospital water network. *Appl. Environ. Microbiol.* **72**, 2428–2438.

Trabelsi, H., Dendana, F., Sellami, A., Sellami, H., Cheikhrouhou, F., Neji, S., Makin, F. & Ayadi, A. 2012 Pathogenic free-living amoebae: epidemiology and clinical review. *Pathol. Biol.* **60**, 399–405.

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