Status of free-living amoebae (*Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris*) in drinking water supplies in Karachi, Pakistan

Farzana Abubakar Yousuf, Ruqaiyyah Siddiqui, Faysal Subhani and Naveed Ahmed Khan

ABSTRACT

The ability of pathogenic free-living amoebae to produce infections is a growing concern. In this study, we investigated the presence of free-living amoebae (*Acanthamoeba* spp., *Naegleria fowleri, Balamuthia mandrillaris*) in drinking water supplies in Karachi, Pakistan. Fifty-two domestic tap water samples were examined. Amoebae were identified by morphological characteristics and polymerase chain reaction. Thirty percent of the examined samples were positive for *Acanthamoeba* spp., 8% for *N. fowleri* while *B. mandrillaris* were not recovered. Additionally we examined secretory IgA antibody to *Acanthamoeba* and *B. mandrillaris*. *Acanthamoeba* antibody prevalence rate was 100% in both males and females, while *B. mandrillaris* antibody prevalence rate was 5.5% in males only (females were negative). Our findings suggest that free-living amoebae are a potential health hazard in domestic water supplies in Karachi, Pakistan.

Key words | *Acanthamoeba*, *Balamuthia mandrillaris*, keratitis, *Naegleria fowleri*, secretory IgA

Farzana Abubakar Yousuf
Ruqaiyyah Siddiqui
Faysal Subhani
Naveed Ahmed Khan (corresponding author)
Department of Biological and Biomedical Sciences,
Aga Khan University,
Stadium Road,
Karachi,
Pakistan
E-mail: *Naveed5438@gmail.com*

INTRODUCTION

Free-living amoebae have gained increasing attention during the past few decades due to their ability to produce serious human and animal infections, which may be fatal (reviewed in Marciano-Cabral & Cabral 2003; Khan 2006; Visvesvara *et al.* 2007). For example, pathogenic *Acanthamoeba* spp. can cause an extremely painful keratitis with blinding consequences that is frequently associated with contact lens use and granulomatous amoebic encephalitis (GAE) that almost always results in death. *Naegleria fowleri* produces primary amoebic meningoencephalitis characterised by headache, stiff neck, altered mental status, seizures and coma leading to death, mostly affecting children and young adults (Tuppeny 2011). Similar to pathogenic *Acanthamoeba* spp., *Balamuthia mandrillaris* is a causative agent of GAE. Immunocompromised and healthy persons, often children and males, are the victims of GAE caused by *B. mandrillaris*. Once the amoebae have invaded the central nervous system, both primary amoebic meningoencephalitis and GAE almost always result in death (Rodriguez-Zaragoza 1994).

Additionally, free-living amoebae play a major role in harbouring many bacterial pathogens, such as *Legionella*, *Pseudomonas*, *Mycobacterium*, *Enterobacter* and *Vibrio*, (Greub & Raoult 2004; Khan 2006; Thomas *et al.* 2010). Several lines of evidence suggest that free-living amoebae help pathogenic bacteria to survive, propagate and colonise ecosystems, thus contributing to their transmission to susceptible hosts (Kilvington 1990; Schuster & Visvesvara 2004; Storey *et al.* 2004; Thomas *et al.* 2004). This is of particular concern in view of (i) increasing numbers of immunocompromised patients, (ii) excessive use of antibiotics and (iii) global warming with increased outdoor activities, added to the ubiquity of these pathogens, leading to increased exposure for susceptible hosts. Developing countries, such as Pakistan, face serious water scarcity and so there is increased public reliance on water storage

doi: 10.2166/wh.2013.112

tanks and wells, which are breeding grounds for free-living amoebae. Despite concerns about amoebic pathogens, there has not been a single report of *Acanthamoeba* keratitis and GAE due to *Acanthamoeba* or *B. mandrillaris* from Pakistan or many other developing countries. The potential of these organisms to cause serious infections makes it imperative to investigate the occurrence of these organisms in our environment. The present study was carried out in Karachi, Pakistan, with the objectives of determining the occurrence and distribution of free-living amoebae (*Acanthamoeba* spp., *N. fowleri, B. mandrillaris*) in the drinking water supplies from different areas of the city and testing for the presence of anti-*A. castellanii* and anti-*B. mandrillaris* antibodies in asymptomatic individuals.

MATERIALS AND METHODS

Study location and water sample collection

Domestic tap water samples ($n = 52$) were collected from various areas of Karachi from May to June 2011. Each sample was collected in a polypropylene bottle and stored at 4 °C until subsequent analysis.

Isolation and identification of *Acanthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris* in water samples

One litre of each water sample was filtered through a sterilised 0.45 μm pore size cellulose filter under vacuum. The filters were cut into four pieces; three pieces were inverted on to 1.5% non-nutrient agar plates containing a lawn of heat-inactivated *E. coli* K-12 laboratory strain HB101 as previously described (Brindley *et al.* 2009). The plates were incubated at 35 °C. Plates were examined using a phase-contrast microscope for the presence of amoebae trophozoites and cysts daily for up to 3 weeks. As a positive control, approx. 10,000 amoebae were added to sterile distilled water in a 1 L bottle and processed as above.

The identity of amoebae was confirmed using polymerase chain reaction (PCR). Briefly, DNA was extracted using Instagene matrix (BioRad) according to the manufacturer's instructions and as described previously (Khan *et al.* 2001). The supernatant containing the DNA was used as a template for PCR and analyzed for the presence of *Acanthamoeba* spp., *N. fowleri* and *B. mandrillaris* with specific primers. For *Acanthamoeba* spp., the genus-specific primer sequence was 5′-TTTGAATTCGCTCCAATAGCGTATATTAA-3′ and 5′-TTTGAATTCAGAAAGAGCTATCAATCTGT-3′ (Kong & Chung 1996). The PCR was performed in a volume of 25 μL containing 1.25 U Taq polymerase (Qiagen), 0.1–1.0 ng DNA, 200 mM dNTPs, 4 mM $MgCl_2$ and 0.5 mM primer. The PCR reaction was performed at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min for 30 cycles, with a final elongation step of 10 min at 72 °C. Amplified DNA was electrophoresed on a 2% agarose gel, stained and visualised under UV illumination. The primer sequence specific for *N. fowleri* was NaeglF192 (3′-GTGCTGAAACCTAGCTATTGTAACTC AGT-5′) and NaeglR344 (5′-CACTAGAAAAAGCAAACC TGAAAGG-3′) (Shakoor *et al.* 2011). The PCR reaction was performed at 95 °C for 30 sec, 63 °C for 30 sec and 72 °C for 30 sec for 35 cycles, with a final elongation of step of 5 min at 72 °C. The length of the amplified fragment was 153 bp. The primer sequence for *B. mandrillaris* was BalaF1451 (5′-TAACCTGCTAAATAGTCATGCCAAT-3′) and BalaR1621 (5′-CAAACTTCCCTCGGCTAATCA-3′) (Qvarnstrom *et al.* 2006) and it amplified a fragment of 171 bp. The PCR reaction was performed at 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec for 35 cycles, with a final elongation step of 5 min at 72 °C. For both *N. fowleri* and *B. mandrillaris* amplified DNA was electrophoresed on a 1.5% agarose gel, stained and visualised under UV illumination.

Enzyme-linked immunosorbent assays to detect secretory IgA against *Acanthamoeba* spp. and *B. mandrillaris* in mucosal secretions

The presence of anti-amoebae secretory IgA (sIgA) in mucosal secretions (saliva obtained from healthy individuals) were determined using an enzyme-linked immunosorbent assays (ELISA) assays as previously described (Leher *et al.* 1998; Alsam *et al.* 2008). Briefly, *A. castellanii* belonging to the T4 genotype (American Type Culture Collection; ATCC 50492) or *B. mandrillaris* (originally isolated from the brain of a mandrill baboon; ATCC 50209) were cultured routinely as described previously (Visvesvara *et al.* 2007). Approximately 50,000 amoebae in 0.2 mL medium were

inoculated per well in 96-well plates at 30 °C for 24 h. Subsequently, wells were air dried, followed by the addition of ice-cold methanol and acetone (1:1) for 60 min at −80 °C. The wells were washed twice with phosphate buffered saline (PBS) containing 0.05% Tween-20 to remove non-adherent amoebae and blocked using 3% bovine serum albumin for 1 h at 37 °C. Saliva samples were collected from 36 asymptomatic individuals and stored at 4 °C until tested. The individuals were residents of the same households where water samples were collected. The saliva samples (100 μL) were added to wells and incubated for 18 h at 4 °C. The following day, amoebae were washed with PBS plus Tween-20 three times and wells were incubated with mouse anti-human IgA antibody conjugated to horseradish peroxidase (1:2,000) (Abcam, Cambridge, UK). The plates were incubated for 60 min at 37 °C and then wells were washed three times as above. Next, 100 μL of substrate solution [0.1% H_2O_2 and 0.1% orthophenylenediamine in citrate-phosphate buffer (0.05 M $Na_2HPO_4.2H_2O$, 0.05 M citric acid, pH 5)] was added. The reactions were allowed to develop for 15 min and finally 100 μL of 3% sulphuric acid was added to stop the reaction. The optical density of each well was determined on a microplate reader (BioRad) at 492 nm. The optical density of amoebae incubated with antibody in the absence of saliva samples were considered as the background. Amoeba prevalence is defined here as saliva reacting with amoebae with an optical density above the background values.

RESULTS

Presence of pathogenic free-living amoebae in drinking water supplies

The domestic tap water samples collected from different locations of Karachi were tested for the presence of pathogenic free-living amoebae by culture and PCR. Out of 52 samples tested in the present study, 15 (30%) were positive for *Acanthamoeba* spp. and 4 (8%) were positive for *N. fowleri*. All samples tested were negative for *B. mandrillaris* (Table 1). Out of 15 *Acanthamoeba* spp.-positive samples, 10 were isolated from drinking water kept in storage tanks, four were isolated from drinking water kept in underground wells and one from a household that used a mixture of a storage tank and a well for their drinking water supplies. All *N. fowleri* recovered in the present study were isolated from drinking water supplies kept in storage tanks.

Table 1 | Distribution of free-living amoebae in domestic tap water supplies in Karachi, Pakistan

Number of samples examined	Source	Amoebae isolated	Positive (%)
44	Tank	*Acanthamoeba* spp.	10 (22.7%)
		N. fowleri	4 (9.1%)
6	Well	*Acanthamoeba* spp.	4 (66.6%)
		N. fowleri	0
2	Tank and well	*Acanthamoeba* spp.	1 (50%)
		N. fowleri	0

ELISA demonstrated the presence of sIgA against *Acanthamoeba* spp. and *B. mandrillaris* in mucosal secretions of residents of Karachi

To determine the presence of anti-*Acanthamoeba* sIgA and anti-*B. mandrillaris* sIgA in saliva, ELISA assays were performed. The use of saliva samples provides a simple, non-invasive approach (samples can be collected with ease) that determines the presence of sIgA antibodies against given antigens, such as those from *Acanthamoeba* spp. and *B. mandrillaris*. Thirty-six samples were obtained from normal asymptomatic individuals, living in Karachi. The ages of the survey subjects ranged from 25 to 65 years. It was interesting that all subjects showed presence of *A. castellanii* antibodies. The overall sIgA antibody prevalence for *A. castellanii* belonging to the T4 genotype was 100%. No significant difference was observed between genders ($p > 0.05$, using paired *t*-test, one tail distribution). The overall sIgA antibody prevalence for *B. mandrillaris* was 5.5% in males, while females were found to be negative for *B. mandrillaris*. A significant difference was observed between the two genders ($p > 0.05$, using paired *t*-test, one tail distribution).

DISCUSSION

Pakistan's population was 180.8 million in 2009; this is projected to reach 208 million by 2020 (WWF 2007; PCRWR

2008). Karachi has a metropolitan area population of 18 million people as a result of rapid urbanisation in Pakistan. It is estimated that more than 30,000 people (including 20,000 children) die every year in Karachi alone because of unsafe water (WWF 2007; PCRWR 2008; Pappas 2011). The situation is further exacerbated by a relatively warm climate, which favours the growth of pathogenic free-living amoebae and other microbes. This is the first report of the occurrence and distribution of pathogenic free-living amoebae in domestic drinking water supplies in Karachi. In our study 38% of domestic drinking water samples tested positive for the presence of pathogenic free-living amoebae, of which 30% contained *Acanthamoeba* spp. and 8% contained *N. fowleri*. These observations are consistent with those reported from other countries. Lorenzo-Morales *et al.* (2005a) identified *Acanthamoeba* in 36.1% (65/180) of tap water samples collected in Jamaica, West Indies. Leiva *et al.* (2008) reported 21% prevalence of *Acanthamoeba* spp. in drinking water samples collected in León municipality, Nicaragua. Jeong & Yu (2005) reported contamination by free-living amoebae of 47% (97/207) of domestic tap water samples in Busan, Korea. Up to 30% of domestic water samples were positive for *Acanthamoeba* in the UK (Kilvington *et al.* 2004), 59.5% in Tenerife, Canary Islands, Spain (Lorenzo-Morales *et al.* 2005a, b) and 2.8% in the USA (Shoff *et al.* 2008); 10% of tap water samples were found to be contaminated by *Acanthamoeba* spp. in Hong Kong (Boost *et al.* 2008). There is a higher prevalence of *Acanthamoeba* spp. in UK tap water than in the USA, which may explain the higher incidence of *Acanthamoeba* keratitis in the UK (Kilvington *et al.* 2004; Shoff *et al.* 2008). Interestingly there is no single case of *Acanthamoeba* keratitis or *Acanthamoeba* granulomatous encephalitis reported in Pakistan, despite the high prevalence of *Acanthamoeba* in the drinking water supplies. This is probably due to lack of awareness and the difficulty in diagnosis. It is most likely that a vast number of infections due to free-living amoebae are undetected and the actual burden is significantly higher, especially in developing countries such as Pakistan, which warrants further investigation. Our results are supported further by our finding that there is 100% *Acanthamoeba* antibody prevalence rate in the healthy individuals examined, with no significant difference being observed between genders, indicating common exposure to these potential pathogens. It also confirms the ubiquitous nature of these organisms that inhabitants of Karachi come across in their routine lives.

The prevalence of anti *B. mandrillaris* sIgA antibody was found to be low, which could be a result of the urban population of Karachi city having limited exposure to soil (housing, farming, gardening, etc., as *B. mandrillaris* is generally found in organic-rich soil (Visvesvara *et al.* 2007)). Only two individuals examined were positive for anti-*B. mandrillaris* sIgA antibody. All drinking water samples tested were negative for *B. mandrillaris,* further confirming these results.

High prevalence of free-living amoebae in drinking water supplies poses serious risks to the public at large. Our results indicate that the situation in the countryside (small cities/villages) must be dire and there is an urgent need to review water management practices. The isolation of pathogenic free-living amoebae from tap water could be due to old plumbing, poor tap water hygiene, environmental settings and the use of water storage tanks and/or wells. The standing water is kept for long periods of time resulting in its exposure to environmental pathogens. All this needs further investigation. In conclusion, the occurrence of *Acanthamoeba* spp. and *N. fowleri* in domestic tap water supplies in Karachi, Pakistan should be considered a potential health risk. The general public should be made aware of the health risks associated with the use of storage tanks and be informed of their appropriate maintenance.

ACKNOWLEDGEMENTS

The authors are grateful for the kind support provided by The Aga Khan University, Pakistan.

REFERENCES

Alsam, S., Jeong, S. R., Dudley, R. & Khan, N. A. 2008 Role of human tear fluid in *Acanthamoeba* interactions with the human corneal epithelial cells. *Int. J. Med. Microbiol.* **298**, 329–336.

Boost, M., Cho, P., Lai, S. & Sun, W. M. 2008 Detection of *Acanthamoeba* in tap water and contact lens cases using polymerase chain reaction. *Optom. Vis Sci.* **85** (7), 526–530.

Brindley, N., Matin, A. & Khan, N. A. 2009 *Acanthamoeba castellanii*: high antibody prevalence in racially and ethnically diverse populations. *Exp. Parasitol.* **121**, 254–256.

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

Jeong, H. J. & Yu, H. S. 2005 The role of domestic tap water in *Acanthamoeba* contamination in contact lens storage cases in Korea. *Kor. J. Parasitol.* **43**, 47–50.

Khan, N. A. 2006 *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol. Rev.* **30**, 564–595.

Khan, N. A., Jarroll, E. L. & Paget, T. A. 2001 *Acanthamoeba* can be differentiated clinically by the polymerase chain reaction and simple plating assays. *Curr. Microbiol.* **43**, 204–208.

Kilvington, S. 1990 Activity of water biocide chemicals and contact lens disinfectants on pathogenic free-living amoebae. *Int. Biodeterior.* **26**, 127–138.

Kilvington, S., Gray, T., Dart, J., Morlet, N., Beeching, J. R., Frazer, D. G. & Matheson, M. 2004 *Acanthamoeba keratitis*: the role of domestic tap water contamination in the United Kingdom. *Invest. Ophthalmol. Vis. Sci.* **45**, 165–169.

Kong, H. H. & Chung, D. I. 1996 PCR and RFLP variation of conserved region of small subunit ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*. *Kor. J. Parasitol.* **34**, 127–134.

Leher, H., Kinoshita, K., Alizadeh, H., Zaragoza, F. L., He, Y. & Niederkorn, J. 1998 Impact of oral immunization with *Acanthamoeba* antigens on parasite adhesion and corneal infection. *Invest. Ophthalmol. Vis. Sci.* **39**, 2337–2343.

Leiva, B., Clasdotter, E., Linder, E. & Winiecka-Krusnell, J. 2008 Free-living *Acanthamoeba and Naegleria* spp. amoebae in water sources of León, Nicaragua. *Rev. Biol. Tropica* **56**, 439–446.

Lorenzo-Morales, J., Lindo, J. F., Martinez, E., Calder, D., Figueruelo, E., Valladares, B. & Ortega-Rivas, A. 2005a Pathogenic *Acanthamoeba* strains from water sources in Jamaica, West Indies. *Ann. Trop. Med. Parasitol.* **99**, 751–758.

Lorenzo-Morales, J., Monteverde-Miranda, C. A., Jiménez, C., Tejedor, M. L., Valladares, B. & Ortega-Rivas, A. 2005b Evaluation of *Acanthamoeba* isolates from environmental sources in Tenerife, Canary Islands, Spain. *Ann. Agric. Environ. Med.* **12**, 233–236.

Marciano-Cabral, F. & Cabral, G. 2003 *Acanthamoeba* spp. as agents of disease in humans. *Clin. Microbiol. Rev.* **16**, 273–307.

Pappas, G. 2011 *Pakistan and water*: new pressures on global security and human health. *Am. J. Public Health* **101**, 786–788.

PCRWR 2008 Annual Report 2005–2006. Part 2. Islamabad, Islamabad, Pakistan: Pakistan Council for Research in Water Resources (PCRWR; 2008). Available at: http://www.pcrwr.gov.pk.

Qvarnstrom, Y., Visvesvara, G. S., Sriram, R. & da Silva, A. J. 2006 Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. *J. Clin. Microbiol.* **44**, 3589–3595.

Rodriguez-Zaragoza, S. 1994 Ecology of free-living amoebae. *Crit. Rev. Microbiol.* **20**, 225–241.

Schuster, F. L. & Visvesvara, G. S. 2004 Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int. J. Parasitol.* **34**, 1001–1027.

Shakoor, S., Beg, M. A., Mahmood, S. F., Bandea, R., Sriram, R., Noman, F., Ali, F., Visvesvara, G. S. & Zafar, A. 2011 Primary amebic meningoencephalitis caused by *Naegleria fowleri*, Karachi, Pakistan. *Emerg. Infect. Dis.* **17**, 258–261.

Shoff, M. E., Rogerson, A., Kessler, K., Schatz, S. & Seal, D. V. 2008 Prevalence of *Acanthamoeba* and other naked amoebae in South Florida domestic water. *J. Water Health* **6**, 99–104.

Storey, M. V., Winiecka-Krusnell, J., Ashbolt, N. J. & Stenstrom, T. A. 2004 The efficacy of heat and chlorine treatment against thermotolerant *Acanthamoebae* and *Legionellae*. *Scand. J. Infect. Dis.* **36**, 656–662.

Thomas, V., Bouchez, T., Nicolas, V., Robert, S., Loret, J. F. & Levi, Y. 2004 Amoebae in domestic water systems: resistance to disinfection treatments and implication in *Legionella* persistence. *J. Appl. Microbiol.* **97**, 950–963.

Thomas, V., McDonnell, G., Denyer, S. P. & Maillard, J. Y. 2010 Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. *FEMS Microbiol. Rev.* **34**, 231–259.

Tuppeny, M. 2011 Primary amoebic meningoencephalitis with subsequent organ procurement: a case study. *J. Neurosci. Nurs.* **43**, 274–279.

Visvesvara, G. S., Moura, H. & Schuster, F. L. 2007 Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol. Med. Microbiol.* **50**, 1–26.

WWF (World Wide Fund for Nature) 2007 Pakistan's waters at risk. Water and health related issues in Pakistan and key recommendations; A special report, WWF – Pakistan, Ferozepur Road, Lahore 54600, Pakistan.

First received 6 June 2012; accepted in revised form 28 December 2012. Available online 6 February 2013