# Presence of Rotavirus and free-living amoebae in the water supplies of Karachi, Pakistan

[1]Farzana Abubakar Yousuf, [2]Ruqaiyyah Siddiqui, [2]Naveed Ahmed Khan*

*[1]Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan;*

*[2]Department of Biological Sciences, Faculty of Science and Technology, Sunway University, Malaysia.*

**Short title: *Rotavirus* and free-living amoebae in drinking water supplies**

*Corresponding address: Department of Biological Sciences, Faculty of Science and Technology, Sunway University, Selangor, 47500, Malaysia. Tel: 60-(0)3-7491-8622. Ext: 7176. Fax: 60-(0)3-5635-8630. E-mail: naveed5438@gmail.com

**Abstract**

*Rotavirus* and pathogenic free-living amoebae are important health problem, especially for developing countries like Pakistan where public has limited access to clean water supplies. Here, we evaluated the prevalence of *Rotavirus* and free-living amoebae (*Acanthamoeba* spp., *Balamuthia mandrillaris, Naegleria fowleri*) in drinking water supplies to Karachi, Pakistan. Six water filtration plants that supply drinking water to the population of Karachi were investigated. Additionally, drinking water samples from households were analyzed for the presence of *Rotavirus* and free-living amoebae. *Rotavirus* was present in 35% of the water samples collected from water filtration plants; however domestic tap water samples had a prevalence of only 5%. Out of 20 water samples from filtration plants, 13 (65%) were positive for *Acanthamoeba* spp., and one (5%) was positive for *B. mandrillaris*. Out of 20 drinking water samples collected from different areas of Karachi, 35% were found to be positive for *Acanthamoeba* spp. *Rotavirus* was detected in 5% of drinking water samples tested. Overall, these findings for the first time showed the presence of Rotavirus, in addition to pathogenic free-living amoebae in drinking water supplies in Karachi that could be an important public health risk for the affected population.

**Key Words:** *Rotavirus, Acanthamoeba, Naegleria, Balamuthia mandrillaris*.

## Introduction

Aquatic environments serve as a reservoir for enteric viruses, free-living amoebae and bacterial pathogens. If untreated effectively, drinking water supplies pose a serious risk to public health.[1-3] Among viruses, species of the genus *Rotavirus* is recognized as one of the leading viruses causing diarrhea in infants and children. *Rotavirus* is a double-stranded RNA virus with icosahedral symmetry. Based upon their genetic and antigenic properties, seven species (A to G) of rotaviruses have been defined. Among these, *Rotavirus A* accounts for more than 90% of gastroenteritis in humans' worldwide.[4] Recently, oral *Rotavirus* vaccine has been introduced in several countries using attenuated rotavirus limiting the number of cases by more than 70%, however it is not part of routine immunization in several countries including Pakistan. The virus is highly transmissible from contaminated surfaces and it could survive up to several weeks or months.[5] Free-living amoebae are common inhabitants of our ecological environment, e.g., soil and water. Given the opportunity and host susceptibility, pathogenic free-living amoebae can cause serious and sometimes fatal infections. These include primary amoebic meningoencephalitis due to *Naegleria fowleri*; granulomatous amoebic encephalitis due to pathogenic *Acanthamoeba* spp., and *Balamuthia mandrillaris*, and a blinding keratitis due to pathogenic *Acanthamoeba* spp.[6] In addition, several lines of evidence suggest that amoebae harbor bacterial and viral pathogens, and help them survive, propagate and colonize, thus contributing to their transmission to susceptible hosts.[7] For example, Verani et al., (8) showed that *A. polyphaga* provides protection to the human adenoviruses in natural environment, the latter in turn can cause a variety of infections. In this regard, infections by amoebae-associated pathogens, as demonstrated by the presence of amoebae as well as Rotavirus, are a potential risk to public health. This is of particular concern as developing

63 countries such as Pakistan face serious fresh water shortage and increased public reliance on
64 water storage tanks and wells. Among a plethora of pathogens, here we investigated the
65 prevalence of *Rotavirus* and representative free-living amoebae (*Acanthamoeba* spp., *B.*
66 *mandrillaris*, *N. fowleri*) in the water filtration plants that supply drinking water to the
67 population of Karachi, and drinking water samples from households. It is hypothesized that
68 *Rotavirus* and free-living amoebae co-exist in the drinking water supplies in a symbiotic
69 relationship.

70 **Material and Methods**

71 ***Study location and water sampling***

72 There are six different water filtration plants that supply drinking water to the city of
73 Karachi, Pakistan and all were analyzed (Fig. 1). These included water samples from Keenjhar
74 Lake and its associated filtration plant (Keenjhar extreme Inlet, K.G. canal outlet, K.B. Feeder
75 inlet point), Gharo filtration plant, Pipri filtration plant, NEK old filtration plant, COD filtration
76 plant and NEK II filtration plant. Water samples were collected between February to July, 2014
77 on a monthly basis. Apart from NEK old filtration plant and NEK II filtration plant, water
78 samples were collected at three points of filtration i.e., inlet, intermediate, and post-treatment
79 samples. From each testing site, two liters of water sample were collected in sterile water
80 bottles and kept at 4$^{o}$C until analyzed, within one week.
81 Two liter water samples were collected from domestic tap water supplies in sterile
82 bottles. A total of 20 samples were collected from different areas of Karachi from May to July
83 2014. All 20 samples were randomly selected from various locations of Karachi city. Each

sample was collected in a polypropylene bottle, and stored at 4ºC until subsequent analysis, within one week.

***Processing of Rotavirus samples***

One liter of water sample was filtered through a sterilized positively charged 47 diameter Sartolon polyamide 0.45µm pore size filter (Sartorius, Goettingen) under vacuum. After filtration, the filter paper was placed in a sterile Petri dish. Viruses were eluted in Tris-glycine buffer containing 1% of beef extract (pH 9.5) with brief horizontal shaking (100 x g) for 20 min at room temperature. The eluate was neutralized with 1 N HCl, and centrifuged for 1 h at 13,000 x g at 4°C. The supernatant was removed and the pellet was resuspended in 200 µl of sterile PBS and stored at -80ºC until analyzed.[9] The extracted virus particles were screened for the presence of *Rotavirus* antigen with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ProSpecT *Rotavirus* Kit, Oxoid Company, UK) as per manufacturer's instructions. The ELISA kit utilizes a polyclonal antibody in a solid-phase sandwich enzyme immunoassay to detect antigen presence in *Rotavirus A* (ProSpecT *Rotavirus* Kit Oxoid). The extracted virus particles or positive and negative controls samples, provided in the kit, were added and incubated with *Rotavirus A* antigen-specific polyclonal antibody, conjugated to horseradish peroxidase. The micro-well plate was incubated at room temperature for 1 h. Next, the wells were washed with wash buffer to remove excess specimen and any unbound enzyme labeled antibody. A chromogen was added and plates incubated for 10 min at room temperature. The presence of specifically-bound enzyme labeled antibody in the micro wells resulted in a color change. The reaction was stopped by adding acid, and absorbance was read at 450 nm. The values ≥0.15 were considered positive for *Rotavirus A* antigen.

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

An additional one liter of water sample was filtered through a sterilized 0.45μm pore size cellulose filter under vacuum. The filters 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.[10] The plates were incubated at 35ºC. Plates were examined using a phase-contrast microscope for the presence of amoebae, daily for up to 3 weeks. As a positive control, approx. 10,000 amoebae were added to sterile distilled water in a 1 liter 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 manufacturer's instructions. The supernatant containing DNA (~1 ng DNA) was used as a template for PCR and analyzed for the presence of *Acanthamoeba* spp., *N. fowleri* and *B. mandrillaris* using specific primers as described previously.[11] The primer sequence for *Acanthamoeba* spp., yielding an amplicon of 910bp [12], *N. fowleri*yielding an amplicon of 153bp, [13] and *B. mandrillaris*yielding an amplicon of 171bp [14] is indicated in Table 1.

**Results and Discussion**

***Enzyme-linked immunosorbent assay (ELISA) demonstrated the presence of Rotavirus antigen in water treatment plant samples and drinking water supplies***

For the first time in Pakistan, water samples from various water filtration plants as well as water samples from drinking water supplies were analyzed for the presence of *Rotavirus A* antigen. Among twenty water samples obtained from six different water filtration plants in Karachi, *Rotavirus* antigen was detected in 7 out of 20 samples (35%) analyzed (Fig. 2) (Table 2). Although *Rotavirus* antigen was found in raw and inlet water samples, but post-treatment

water samples from Keenjhar Lake filtration plant, Gharo filtration plant, Pipri filtration plant, NEK old filtration plant, and NEK II filtration plant did not show the presence of *Rotavirus* antigens. However, water samples collected from COD filtration plant showed the presence of *Rotavirus* antigens in inlet, intermediate, as well as post-treatment water samples. Twenty tap water samples from households were also collected from drinking water supplies from different regions of Karachi and analyzed. *Rotavirus* antigen was detected in one water sample out of 20 (5%) drinking water samples tested (Table 2). When the data was grouped for each plant, it was worthy to note that two plants out of six were able to clean the *Rotavirus* contamination and one out of six provides an ineffective treatment, while three plants out of six treat *Rotavirus*-free incoming water. Thus, this data may explain the lower prevalence of *Rotavirus* antigen in tap water as compared to the water collected from the treatment plants.

***Presence of free-living amoebae in samples from water filtration plants and drinking water supplies of Karachi***

The water samples collected from six water treatment plants were tested for the presence of free-living amoebae (*Acanthamoeba* spp., *N. fowleri* and *B. mandrillaris*) using PCR. Out of twenty water samples from filtration plants, 13 (65%) were positive for *Acanthamoeba* spp., and 1 (5%) was positive for *B. mandrillaris* (Table 2). All samples tested negative for *N. fowleri*. Out of 20 drinking water samples collected from different areas of Karachi, 7 (35%) were positive for *Acanthamoeba* spp., however all drinking water samples were negative for *N. fowleri* and *B. mandrillaris* (Table 2).

Drinking water supplied to the population of Karachi is sourced from Keenjhar Lake and carried to water treatment plants for setting, filtration and chlorination. Subsequently, water

is distributed to the local population of Karachi. Keenjhar Lake is situated in Thatta district, Sindh and is one of the main sources of water (900 cusecs per day) through which drinking water is supplied to Karachi, albeit intermittently. It is estimated that >30,000 people (out of which 20,000 are children) die every year due to diarrheal diseases in Karachi alone because of unsafe water.[15-17] Among diarrheal pathogens, *Rotavirus* is a leading cause of morbidity and mortality in children.[18-20] For the first time in Pakistan, here we showed the presence of *Rotavirus* in 35% of water samples collected from water filtration plants as well as 5% of water samples collected from drinking water supplies. In comparison, *Rotavirus* was detected in 1.4% of drinking water samples analyzed in Pretoria, South Africa, and 11.8% of water samples collected from water purification plants,[21] 2% of water samples in Slovenia,[22] while in China, *Rotavirus* was detected in 34.6% samples of raw water drawn from the source; 11.7% of treated water samples and 22.4% tap water samples from different areas of Beijing,[23] albeit different methodologies were used to detect *Rotavirus*. *Rotavirus* was detected in up to 62.5% of water samples collected from filtration plants,[18] while 37.5% water samples were positive for *Rotavirus* in drinking water samples in Columbia; 48.1% of water samples tested were positive for *Rotavirus* in Ghana.[24] Notably, our study used ELISA to detect *Rotavirus* antigen and it is limited to water samples collected from filtration plants as well as households that were supplied treated water by the local Karachi Water and Sewage Board. This excludes many areas slums and other areas of Karachi. A large disadvantaged population of Karachi relies on water through tanker and other sources that is often untreated, which is the subject of future studies. Future studies should investigate water samples supplied to larger population of Karachi as well as seasonal distribution of microbial contaminants, which will provide accurate account of prevalence of pathogenic microbes in the drinking water supplies of Karachi. For example,

several lines of investigations showed an association between the occurrence of *Rotavirus* infection and weather conditions. For example, *Rotavirus* infection was observed to be high in the winter season, compared with the summer season in the USA,[19] Japan,[25] northern Asian regions,[20] temperate regions in Australia[26] and Europe.[27] In moderate climate regions, a peak in *Rotavirus* infection was often observed in colder and drier months of the year.[28] A comprehensive study on the seasonal distribution of *Rotavirus* in drinking water supplies of Karachi and the number of *Rotavirus*-associated diarrheal cases in local hospitals throughout the year will determine the effectiveness of present water treatment practices.

Our study also showed that up to 65% water samples were positive for *Acanthamoeba* spp. and 5% of water samples were positive for *B. mandrillaris* from water collected from filtration plants, while 35% of drinking water samples were positive for *Acanthamoeba* spp. Notably, when the contamination was already present in the incoming water (three plants out of six), none of the analyzed plants were able to reduce it. Moreover two plants out of six (i.e., COD and Pipri, Table 2) showed *Acanthamoeba* spp. and/or *B. mandrillaris* contamination in the out coming, but not in the incoming water. These findings suggest source of amoebae contamination, post-treatment, which should be investigated in further studies. This is in accordance to the high prevalence of *Acanthamoeba* spp. (35%) found in the tap water samples. These results are consistent with previous findings which showed the prevalence of free-living amoebae at 38% of water samples tested.[11] For example, in Sivas, Turkey, free-living amoebae were found in 30% of drinking water samples,[29] while water samples in Nicaragua had a prevalence of 21% for *Acanthamoeba* spp. in drinking water supplies.[30] *Acanthamoeba* are known to predate and/or harbour viruses, bacteria, protists and often referred to as the Trojan horse of the microbial world.[7] In support, recent studies tested the interaction between *A.*

198 *polyphaga* and Human Adenovirus to determine whether the amoeba played a role in protecting
199 the internalized viruses from chemical disinfection. The results revealed that when amoeba and
200 Human Adenovirus were co-cultured, infectious virus was more resistant to disinfection
201 suggesting that *A. polyphaga* is providing protection for the Human Adenovirus. Based on these
202 findings, it is tempting to speculate an association between *Acanthamoeba* and *Rotavirus*.
203 Notably, recent studies suggest that *Rotavirus* internalization of *A. castellanii* occurs via
204 infected mammalian cells only, but not freely suspended virus.[31] The precise *Rotavirus*
205 association with *Acanthamoeba* and the potential implications of amoeba to harbour virus
206 during its cyst stage to protect against water disinfection strategies as well as help their
207 transmission to susceptible population is an interesting hypothesis which is the subject of future
208 studies. Moreover, it can be speculated that viruses ingested by *Acanthamoeba* can be taken up
209 by protists into biofilms and survive within this protected niche environment. Upon
210 environmental stimuli, viruses may escape from the biofilm, presenting a potential threat. It is
211 also possible that *Rotavirus* replicate within the infected protist, or that multiplication of protists
212 may lead to increased progeny of the virus, thus increasing the potential for virus spread.
213 Overall, this study has underlined the need for additional investigations of aquatic
214 environments, in addition to drinking water supplies to larger population of Karachi, to detect
215 *Rotavirus* strains and free-living amoebae circulating in the community, their seasonal
216 distribution and possible association during various stages of the life cycle of *Acanthamoeba*.
217 The effective management of public water supplies and the implementation of proper
218 precautionary regulatory actions will assist in the prevention of serious diseases caused by
219 *Rotavirus* and pathogenic protists or due to their symbiotic hyperparasitic roles.
220

**Acknowledgements:** The authors are grateful for the kind support provided by Aga Khan University, Pakistan and Sunway University, Malaysia. The authors are grateful to Syed Usman Bin Mahmood, Atteeba Manzar, Ammar Alam for technical support.

**Competing interests:** The authors declare no competing interests.

**Authors' Contribution:** NAK conceived the study. YFA and RS designed the experiments. All experiments were performed by YFA under the supervision of RS and NAK. YFA performed analyses and interpretations. YFA wrote the first draft of the manuscript. RS and NAK corrected the manuscript. All authors approved the manuscript.

**Ethical approval:** Not applicable.

## References

1. Fong TT, Lipp EK. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiol Mol Biol Rev. 2005;69:357-71.

2. World Health Organization. Water sanitation health: Burden of disease and cost-effectiveness estimates. Available at: http://www.who.int/water_sanitation_health/diseases/burden/en/

3. Centers for disease control and prevention. Water-related diseases and contaminants in public water systems. Available at http://www.cdc.gov/healthywater/drinking/public/water_diseases.html

4. Desselberger U. Rotaviruses. Virus Res. 2012;190:75-96.

5. Dennehy PH. Rotavirus infection: an update on management and prevention. Adv Pediat. 2012;59:47-74.

6. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*. FEMS Immunol Med Microbiol. 2007;50:1-26.

7. Khan NA. *Acanthamoeba*: Biology and Pathogenesis. Second Edition, Caister Academic Press, Linton, Cambs, UK. 2015. p344.

8. Verani M, Di Giuseppe G, Tammaro C, Carducci A. Investigating the role of *Acanthamoeba polyphaga* in protecting Human Adenovirus from water disinfection treatment. Eur J Protistol. 2016;54:11-18.

9. Steyer A, Torkar KG, Gutiérrez-Aguirre I, Poljšak-Prijatelj M. High prevalence of enteric viruses in untreated individual drinking water sources and surface water in Slovenia. Int J Hyg Environ Health 2011;214:392-8.

10. Brindley N, Matin A, Khan NA. *Acanthamoeba castellanii*: high antibody prevalence in racially and ethnically diverse populations. Exp Parasitol. 2009;121:254-6.

11. Yousuf FA, Siddiqui R, Subhani F, Khan NA. Status of free-living amoebae (*Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris*) in drinking water supplies in Karachi, Pakistan. J Water Health 2013;11:371-5.

12. Kong HH, Chung DI. 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. 1996;34:127-34.

13. Shakoor S, Beg MA, Mahmood SF, Bandea R, Sriram R, Noman F, Ali F, Visvesvara GS, Zafar A. Primary amebic meningoencephalitis caused by *Naegleria fowleri*, Karachi, Pakistan. Emerg Infect Dis. 2011;17:258-61.

14. Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. J Clin Microbiol. 2006;44:3589-95.

15. WWF. Pakistan's waters at risk Water and health related issues in Pakistan and key recommendations. 2007. p1-33. A special report, WWF — Pakistan, Ferozepur Road, Lahore — 54600, Pakistan.

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

17. Pappas G. Pakistan and water: new pressures on global security and human health. Am J Pub Health 2011;101:786-8.

18. van Zyl WB, Page NA, Grabow WO, Steele AD, Taylor MB. Molecular epidemiology of group A rotaviruses in water sources and selected raw vegetables in southern Africa. Appl Environ Microbiol 2006;72:4554-60.

19. Gutiérrez-Aguirre I, Steyer A, Boben J, Gruden K, Poljsak-Prijatelj M, Ravnikar M. Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. J Clin Microbiol. 2008;46:2547-54.

20. He XQ, Cheng L, Zhang DY, Li W, Xie XM, Ma M, Wang ZJ. First molecular detection of group A rotaviruses in drinking water sources in Beijing. Chin Bull Environ Contam Toxicol. 2009;83:120-4.

21. Gutiérrez MF, Alvarado MV, Martínez E, Ajami NJ. Presence of viral proteins in drinkable water sufficient condition to consider water a vector of viral transmission? Water Res. 2007;41:373-8.

22. Dongdem JT, Adjimani J, Armah G. Detection and characterization of human rotavirus in tap water by multiplex RT-PCR. J Medic Med Sci. 2010;1:223-30.

23. Glass RI, Kilgore PE, Holman RC, Jin S, Smith JC, Woods PA, Clarke MJ, Ho MS, Gentsch JR. The epidemiology of rotavirus diarrhea in the United States: surveillance and estimates of disease burden. J Infect Dis. 1996;174:S5-11.

24. Konno T, Suzuki H, Katsushima N, Imai A, Tazawa F, Kutsuzawa T, Kitaoka S, Sakamoto M, Yazaki N, Ishida, N. Influence of temperature and relative humidity on human rotavirus infection in Japan. J Infect Dis. 1983;147:125-8.

25. Bresee J, Fang ZY, Wang B, Nelson EA, Tam J, Soenarto Y, Wilopo SA, Kilgore P, Kim JS, Kang JO, Lan WS, Gaik CL, Moe K, Chen KT, Jiraphongsa C, Ponguswanna Y, Nguyen VM, Phan V T, Le TL, Hummelman E, Gentsch JR, Glass R. Asian Rotavirus Surveillance Network. First report from the Asian Rotavirus Surveillance Network. Emerg Infect Dis 2004;10:988-95.

26. Bishop RF, Masendycz PJ, Bugg HC, Carlin JB, Barnes GL. Epidemiological patterns of rotaviruses causing severe gastroenteritis in young children throughout Australia from 1993 to 1996. J Clin Microbiol. 2001;39:1085-91.

27. Cook SM, Glass RI, LeBaron CW, Ho MS. Global seasonality of rotavirus infections. Bull World Health Org. 1990;68:171-7.

28. Ansari SA, Springthorpe VS, Sattar SA. Survival and vehicular spread of human rotaviruses: possible relation to seasonality of outbreaks. Rev Infect Dis. 1991;13:448-61.

29. Ozçelik S, Coşkun KA, Yünlü O, Alim A, Malatyal E. The prevalence, isolation and morphotyping of potentially pathogenic free-living amoebae from tap water and environmental water sources in Sivas. Turk Parazitol Derg. 2012;36:198-203.

30. Leiva B, Clasdotter E, Linder E, Winiecka-Krusnell J. Free-living *Acanthamoeba* and *Naegleria* spp. amebae in water sources of León, Nicaragua. Rev Biol Trop. 2008;56:439-46.

31. Alotaibi MA. Internalisation of enteric viruses by *Acanthamoeba castellanii*, via ingestion of virus-infected mammalian cells. Food Environ Virol. 2011;3:109-14.

**Figure legends**

Figure 1. Map showing water collection sites for water filtration, supplying water to the city of Karachi, Pakistan.

Figure 2. Water samples from different filtration plants (Keenjhar, Gharo, Pipri, NEK old, COD, and NEK II filtration plants) were collected between February to July, 2014 and tested for the presence of *Rotavirus A* antigen as described in Materials and Methods. The presence of *Rotavirus* antigen was detected using a commercially available enzyme-linked immunosorbent

assay (ELISA) kit (ProSpecT *Rotavirus* Kit, Oxoid Company, UK) as per manufacturer's instructions. The ELISA kit utilizes a polyclonal antibody in a solid-phase sandwich enzyme immunoassay to detect *Rotavirus A* antigen (ProSpecT *Rotavirus* Kit Oxoid). The extracted virus particles or positive and negative controls samples, provided in the kit, were added and incubated with *Rotavirus* antigen-specific polyclonal antibody, conjugated to horseradish peroxidase. The values ≥0.15 were considered positive for *Rotavirus* antigen.