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ORIGINAL PAPER

Isolation and molecular characterization of potentially pathogenic *Acanthamoeba* genotypes from diverse water resources including household drinking water from Khyber Pakhtunkhwa, Pakistan

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Abstract *Acanthamoeba*, an opportunistic protozoan pathogen, is ubiquitous in nature, and therefore plays a predatory role and helps control microbial communities in the ecosystem. These *Acanthamoeba* species are recognized as opportunistic human pathogens that may cause blinding keratitis and rare but fatal granulomatous encephalitis. To date, there is not a single report demonstrating *Acanthamoeba* isolation and identification from environmental sources in Pakistan, and that is the aim of this study. *Acanthamoeba* were identified by morphological characteristics of their cysts on non-nutrient agar plates seeded with *Escherichia coli*. Additionally, the polymerase chain reaction (PCR) was performed with genus-specific primers followed by direct sequencing of the PCR product for molecular identification. Furthermore, our PCR and sequencing results confirmed seven different pathogenic and nonpathogenic genotypes, including T2–T10, T4, T5, T7, T15, T16, and T17. To the best of our knowledge, we have identified and isolated *Acanthamoeba* sp., for the first time, from water resources of Khyber Pakhtunkhwa, Pakistan. There is an urgent need to address (1) the pathogenic potential of the identified genotypes and (2) explore other environmental sources from the country to examine the water quality and the current status of *Acanthamoeba* species in Pakistan, which may be a potential threat for public health across the country.

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Introduction

Acanthamoeba is a free-living protozoan pathogen widely distributed in nature. They can be found in soil, dust, air, drinking water; tap, fresh, coastal, and bottled mineral water; seawater, beach sands, sewage, flowerpot soils, home aquaria, humidifiers, heating, ventilation air conditioning units, hospitals, dental and dialysis units, contact lens solutions and eyewash stations, laboratory distilled water bottles, and chlorinated swimming pools (Auran et al. 1987; Stehr-Green et al. 1989; Badenoch et al. 1994; Walker 1996; Sriram et al. 2008). In addition, they have been isolated from vegetables, fishes, reptiles, amphibians, dogs, monkeys, birds, pulmonary secretions, maxillary sinuses, and stool samples (Visvesvara et al. 2007) and are known to be one of the most ubiquitous of organisms. *Acanthamoeba* have adapted to withstand diverse environmental conditions by switching their phenotype. Under harsh environmental conditions such as lack of nutrients, high temperatures, and high osmolarity, *Acanthamoeba* encyst to a resistant cyst. However, under favorable conditions,

Acanthamoeba cysts excyst to vegetative infective trophozoites (Walker 1996; Sriram et al. 2008).

Acanthamoeba castellanii consists of both pathogenic and nonpathogenic strains. Given the correct access and host conditions, pathogenic *Acanthamoeba* can cause serious human diseases such as eye keratitis or a rare and life-threatening *Acanthamoeba* granulomatous encephalitis (Auran et al. 1987; Stehr-Green et al. 1989; Badenoch et al. 1994; Di Gregorio et al. 1991). Given the free-living nature of these amoebae, we likely encounter *Acanthamoeba* during our normal everyday life. *Acanthamoeba* cases may go undiagnosed in developing countries due to their lack of awareness, expertise, and difficulty in diagnosis. Most disturbingly though is that there is a lack of effective chemotherapeutic treatment for the infection. *A. castellanii* has been classified into 17 different genotypes (T1–T17) (Schuster and Visvesvara 2004; Nuprasert et al. 2010) and has been isolated from environmental sources worldwide, including Asian countries such as Korea, Japan, and Thailand (Choi et al. 2009; Edagawa et al. 2009; Nuprasert et al. 2010), but there is not a single report available in literature regarding *A. castellanii* isolation or its presence in Pakistan. To the best of our knowledge, this is the first study demonstrating and molecularly characterizing *A. castellanii* genotypes in water resources of Khyber Pakhtunkhwa (KPK), Pakistan.

Materials and methods

Cultures of *Acanthamoeba* and bacteria

A clinical isolate of *A. castellanii* belonging to the T4 genotype isolated from a keratitis patient (ATCC 50492) was used as a positive control. Cultures potentially containing *Acanthamoeba* were grown without shaking in 15 ml of PYG medium (protease peptone 0.75 % (w/v), yeast extract 0.75 % (w/v), and glucose 1.5 % (w/v)) in T75 tissue culture flasks at 30 °C as previously described (Raza et al. 2012). Gram-negative bacteria *Escherichia coli* K12 (HB101), a noninvasive laboratory strain, were also used as food for the *Acanthamoeba*, in this study. Bacteria were grown in Luria–Bertini (LB) medium, containing 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, and 1 % (w/v) NaCl overnight, as described previously (Matin and Jung 2011).

Morphological identification

Environmental samples collection and *Acanthamoeba* isolation using plating assays

We conducted a survey for *A. castellanii* isolation in 15 representative cities of KPK, Pakistan (Fig. 1). Water samples were collected from different sources, i.e., household drinking water (borings, wells, and municipal supplies), rivers, canals, sewage water, and air conditioning units water in KPK, Pakistan (34.952682°N, 72.331120°E), from January 2011 to December 2011. Samples were collected in sterile polypropylene bottles, labeled with date, time, and place of collection. For *A. castellanii* isolation, plating assays were performed as described previously (Lorenzo-Morales et al. 2005). Briefly, *E. coli* were grown in LB medium overnight and then heat killed. The heat-killed *E. coli* were poured onto non-nutrient agar plates and left for 2–3 min. Excess culture medium was poured off, and plates were left to dry. Water samples (500 ml) were filtered through sterile nitrocellulose membrane (pore size: 0.2 μm), and these filters were placed upside down on the 1.5 % non-nutrient agar plates, with a lawn of killed *E. coli*, and these plates were incubated at 30 °C. These plates were kept sealed in plastic bags in a humidified atmosphere to prevent drying and examined with an inverted microscope at ×400. Following the detection of amoebae feeding on *E. coli* on agar, portions of the agar that contained these amoebae were excised and transferred onto fresh *E. coli*coated plates. After the amoebae of interest had migrated away from fungal and other contaminants, they were transferred in agar cores to fresh *E. coli*-coated agar plates. Next, the plates were monitored microscopically for up to 2 weeks for growth of *A. castellanii* trophozoites or for the presence of cysts.

Molecular identification

DNA extraction

A. castellanii were scraped from the agar plates, and DNA extraction was performed by modifying our previous protocol (Matin et al. 2006; Zhang et al. 2004). Briefly, amoebae were washed 3× in PBS (pH 7.4) and resuspended in 500 μl of cell lysis buffer (100 mM KCl, 40 mM Tris, 25 mM $MgCl_2$, 1 % Tween-20, and 0.1 mg/ml proteinase K). After gentle mixing by inversion, the lysates were incubated at 56 °C for 3 h. These samples were chilled on ice for 5 min and were extracted with equal volumes of phenol–chloroform (1:1). After extraction, the aqueous and organic phases were separated by centrifugation at 15,000 rpm for 15 min at room temperature (RT). Next, the supernatant was extracted with an equal volume of chloroform–isoamyl alcohol (24:1). After centrifugation at 15,000 rpm, the upper aqueous phase was collected and DNA precipitated by adding 1/10 volume of 10 M ammonium acetate plus two volumes of cold absolute ethanol and kept at −20 °C overnight. The DNA was pelleted by centrifugation at 15,000 rpm, after washing with 100 μl of 70 % ethanol, then dried at RT, dissolved in 300 μl of double-distilled water, and stored at −20 °C until used.

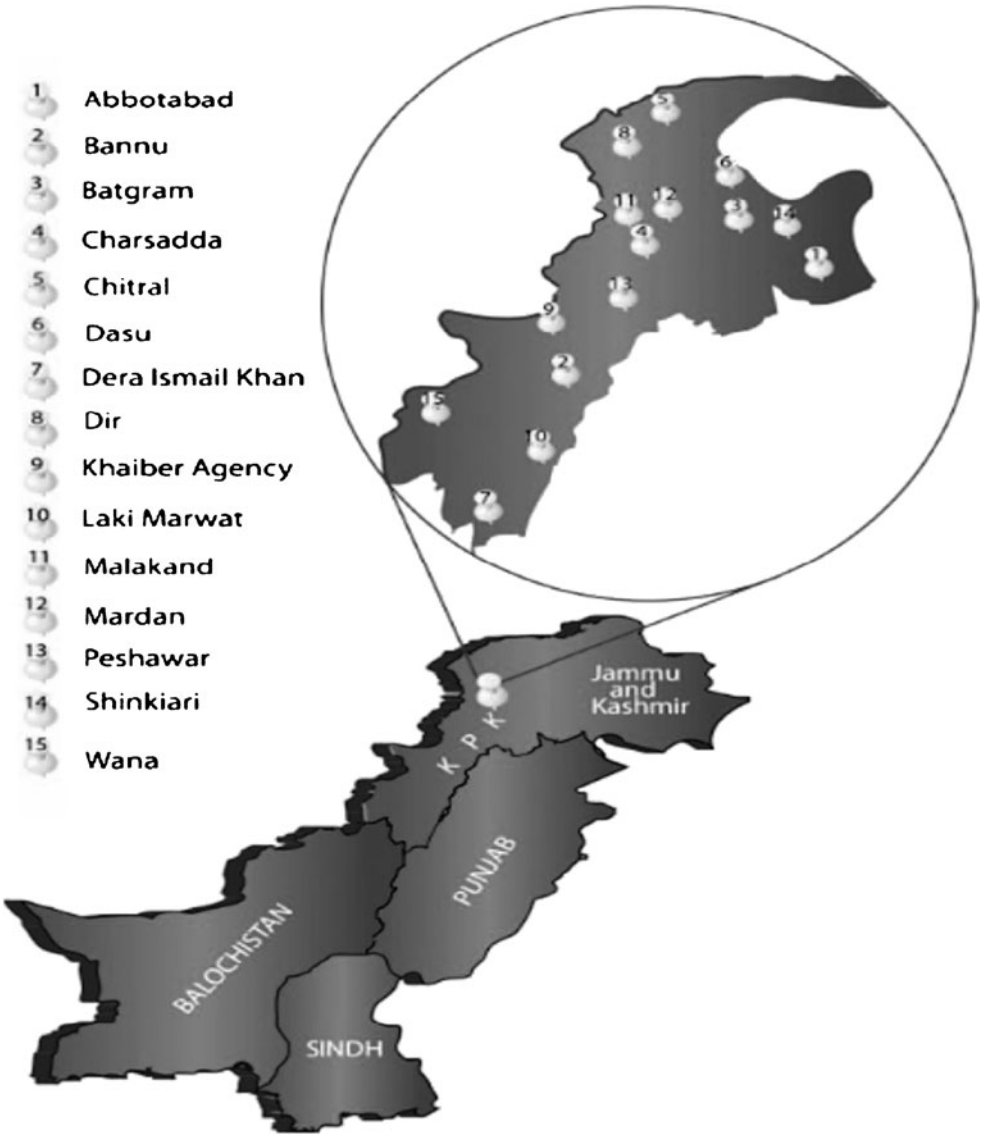

Fig. 1 **a** Map showing sampling sites in the targeted province of "Khyber Pakhtunkhwa" in Pakistan

Identification of *Acanthamoeba* using PCR amplifications and genotyping

The DNA amplification was performed using genus-specific primers as described previously (Matin et al. 2006; Booton et al. 2002). Primer pairs include the forward primer JDP1 (5-GGCCCAGATCGTTTACCGTGAA) and the reverse primer JDP2 (5-TCTCACAAGCTGCTAGGGAGTCA). PCR reactions were performed in a 20 μl volume, containing 1.25 U Taq polymerase (Qiagen), 0.2–0.4 μg DNA, 200 μM dNTPs, 2 mM $MgCl_2$, and 2 μM primer, at 95 °C for 5 min for 1 cycle, 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min for 35 cycles and a final elongation step of 10 min at 72 °C. Amplified DNA was electrophoresed on 2 % agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. After purification, direct sequencing of each PCR product was performed with an automated fluorescence sequencing system (3130 Genetic Analyser, model 627–0040; Applied Biosystems, Tokyo, Japan), with the conserved primer 892C(5′-GTCAGAGGTGAAATTCTTGG) to determine the primary DNA sequence of DF3 of *Rns*. The DF3 sequence nomenclature was used in this study as described previously (Booton et al. 2002).

Results and discussion

Microscopic identification of *Acanthamoeba* from water samples on non-nutrient agar plates

Based on morphological characteristics of trophozoites or cysts, amoebae from the genus *Acanthamoeba* were

identified as positive (for growth of amoeba) from water samples on non-nutrient agar plates after 7–14 days, when inspected visually under inverted microscope. Isolated amoebae were maintained and purified by periodically cutting out a small piece of agar containing trophozoites or cysts and transformed onto a fresh agar plate with a bacterial lawn. Overall, 35 of the 38 samples (92 %) were positive for the growth of amoeba on non-nutrient agar plates (only represented samples were shown in Fig. 2).

PCR confirms the presence of *Acanthamoeba* in water samples recovered from non-nutrient agar plates

To further confirm the presence of *Acanthamoeba* population on non-nutrient agar plates, DNA was isolated from scrapings obtained from plates and used for PCR reactions using *Acanthamoeba* genus specific JDP1 and JDP2 primers. A single PCR product of ~500 bp was obtained, confirming the presence of *Acanthamoeba* DNA (Fig. 3). Our results

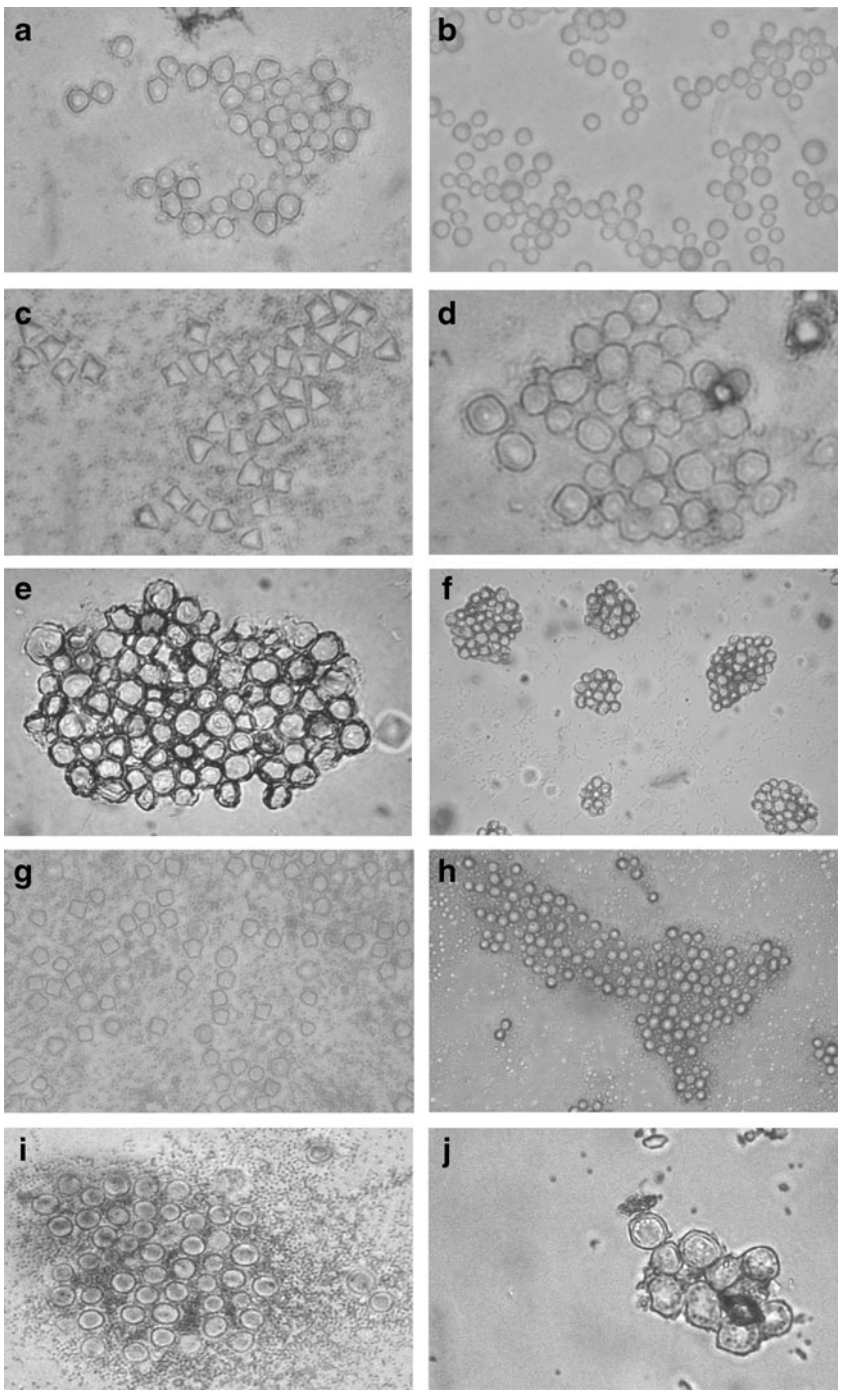

Fig. 2 *Acanthamoeba* cysts (×400) on non-nutrient agar plates when observed under inverted microscope. Water samples were filtered and inoculated on non-nutrient agar plate lawn with *E. coli* as described in Materials and methods. Plates were observed for outgrowth up to 14 days, and images were taken. The representative samples **a** CHCW1, **b** KAW3, **c** PSHW1, **d** KACW1, **e** CHCW4, **f** KAW4, **g** PSHAC1, **h** PSHCW4, **i** PSHSW7, and **j** LMW6 were shown here

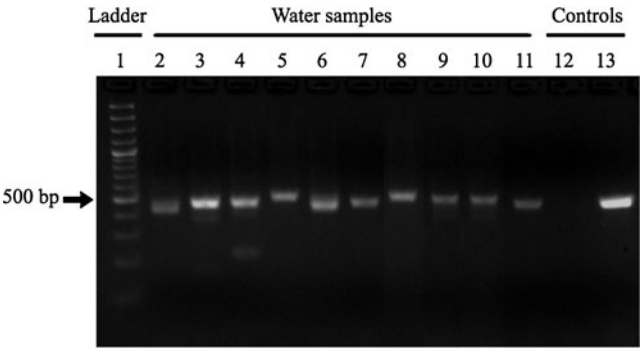

Fig. 3 PCR analysis of the amoeba isolated from the water samples. To confirm the presence of *Acanthamoeba* populations, DNA were isolated from amoeba recovered from non-nutrient agar plates after 7–14 days and used for PCR analysis using *Acanthamoeba* genus-specific primers pairs JDP1 and JDP2 as described in Materials and methods. PCR products were found about 500 bp in all samples, confirming the presence of *Acanthamoeba*. *Lane 1* 100 bp DNA ladder, *Lane 2* KACW1, *Lane 3* KAW3, *Lane 4* PSHW1, *Lane 5* CHCW1, *Lane 6* CHCW4, *Lane 7* LMW6, *Lane 8* KAW4, *Lane 9* PSHAC1, *Lane 10* PSHCW4, *Lane 11* PSHSW7, *Lane 12* negative control, and *Lane 13* positive control

revealed 15 of 35 (42 %) samples were successfully amplified (only represented samples were shown in Fig. 3). *A. castellanii* (T4 genotype; ATCC 50492) was used as a positive control.

Pathogenic and nonpathogenic *Acanthamoeba* genotypes were identified by sequencing

To clarify the relation between the *Acanthamoeba* DNA found in water samples, phylogenetic analysis was performed on variable region of 18S rDNA. After sequencing, 10 out of 15 (66.6 %) partial sequences were obtained and submitted to GenBank (Table 1). We therefore isolated seven strains belonging to both pathogenic [T2–T10 (GenBank accession no. KC203589), T4 (GenBank accession nos. KC203590, KC493777, KC493776, and KC4 93774), T5 (GenBank accession no. KC493773), and T15 (GenBank accession no. KC203588)] and nonpathogenic genotypes [T7 (GenBank accession no. KC493775), T16 (GenBank accession no. KC203592), and T17 (GenBank accession no. KC203591)] of *A. castellanii* from water resources of KPK, Pakistan.

The present study assessed the presence of *Acanthamoeba* from different water sources of KPK, Pakistan. Here for the first time, we isolated various pathogenic (T2–T10, T4, T5, T15) and nonpathogenic (T7, T16, and T17) *A. castellanii* genotypes from water resources (especially household drinking water) of KPK, Pakistan. In support, we have shown recently that the tribal population of KPK has a high titer level of anti-*Acanthamoeba* antibodies (Matin et al. 2012) as compared to

Table 1 *Acanthamoeba* genotypes identified in different water resources from the representative areas of Khyber Pakhtunkhwa, Pakistan

Sample code	Sources	Sampling area	NNA culture	PCR	Specie	Genotypes	GenBank accession no.
ABD11	Municipal drinking water	Abbottabad	+	+		–	–
BGM8	Stream water	Batgram	+	+		–	–
BNUW3	Boring (drinking water)	Bannu	+	+		–	–
CHCW1	Canal water	Charsada	+	+		T17	KC203591
CHCW4	Canal water	Charsada	+	+		T16	KC203592
CHT9	Stream water	Chitral	+	–		–	–
DASUW6	Boring (drinking water)	Dasu	–	–		–	–
DIKW9	Boring (drinking water)	Dera Ismail Khan	+	+		–	–
DIRW12	Boring (drinking water)	Dir	+	+		–	–
KACW1	Canal water	Khyber Agency	+	+	*A. lenticulata*	T5	KC493773
KAW3	Boring (drinking water)	Khyber Agency	+	+		T15	KC203588
KAW4	Boring (drinking water)	Khyber Agency	+	+		T4	KC493777
LMW6	Boring (drinking water)	Laki Marwat	+	+		–	–
MK7	Municipal drinking water	Malakand	+	–		–	–
MRD5	Tube well (drinking water)	Mardan	+	–		–	–
PSHW1	Boring (drinking water)	Peshawar	+	+		T2–T10	KC203589
PSHW1	Boring (drinking water)	Peshawar	+	+		T4	KC203590
PSHAC1	Air condition water	Peshawar	+	+		T4	KC493776
PSHCW4	Canal water	Peshawar	+	+	*A. astronyxis*	T7	KC493775
PSHSW7	Sewage water	Peshawar	+	+		T4	KC493774
SKIW5	Boring (drinking water)	Shinkiari	+	+		–	–
WA6	Tap water (drinking water)	Wana	+	–		–	–

the rest of the country. Infections due to *Acanthamoeba* have increased worldwide over the years, which is likely due to the presence of *Acanthamoeba* in the natural environment (Rezaeian et al. 2008; Liang et al. 2010) and, thus, their having direct contact with humans in everyday life (Anisah et al. 2005; Ledee et al. 2009). This is likely due to increasing numbers of contact lens wearers, increasing populations of immunocompromised patients, and global warming (Hunter 2003; Marciano-Cabral and Cabral 2003; Ibrahim et al. 2009). Despite the free-living nature of *Acanthamoeba* and its widespread presence in the environment, there has not been a single reported case of keratitis or encephalitis infections from Pakistan so far, which may be attributed to a lack of expertise and awareness among the medical community, poor diagnosis, and poor public health systems.

Amoebic research has been well-established in developed countries, but in developing countries such as Pakistan, amoebic research has been under studied; even though, we still do not have the basic necessities of life, i.e., availability of potable drinking water, food, air, and environment in particular. We have many concerns about public health across the country. Keeping in mind the free-living nature of the organisms, we come across *Acanthamoeba* in our normal everyday life. It has been experienced in the majority of cases that *Acanthamoeba* keratitis is often misdiagnosed as herpes simplex virus or adenovirus infections (Hammersmith 2006). In addition, our major concern is unawareness, lack of expertise on this pathogen, and a polluted environment; we could expect more *Acanthamoeba* cases from Pakistan than the rest of the world. But to date, there is no documented evidence of such infections from Pakistan. Overall, there is an urgent need for more detailed knowledge about the distribution of *Acanthamoeba* genotypes in different environmental sources of Pakistan and their direct and indirect virulence factors. These findings will be useful in establishing a baseline for the future for new research and clinicians to be aware of this amoeba and its infections. This report will be also useful to make our community aware of the pathogens, which may cause life-threatening infections in humans and animals.

It is well-established that *Acanthamoeba* facilitate bacterial (Sriram et al. 2008) and viral (Scheid and Schwarzenberger 2012) transmission and may provide protection against the human immune system. The ability of *Acanthamoeba* to resist harsh conditions (such as extreme temperatures, pH, and osmolarity), especially during their cyst stage (Sriram et al. 2008), suggests their usefulness as bacterial vectors. In particular, *Acanthamoeba* cysts are notoriously resistant to chlorine (a key and sometimes only compound used in cleaning water systems). This poses clear challenges in eradicating bacterial pathogens from public water supplies, especially in developing countries (Visvesvara et al. 2007; Lorenzo-Morales et al. 2006; Magliano et al. 2009; Chang et al. 2010; Thomas et al. 2010). In addition, *Acanthamoeba*–bacteria interactions also affect bacterial virulence; i.e., *Legionella pneumophila* grown within *Acanthamoeba* exhibited increased motility, virulence, and drug resistance compared with axenically grown *L. pneumophila* (Greub and Raoult 2002). There is a possible danger that *Acanthamoeba* may be used as breeding ground for different pathogenic bacteria, which could be a potential threat to human health.

The majority of human infections (keratitis, non-keratitis infections such as *Acanthamoeba* granulomatous encephalitis and cutaneous infections) due to *Acanthamoeba* have been associated with the T4 genotype. For example, more than 90 % of keratitis cases have been linked with this genotype, which is the most likely genotype to express a strong virulence against human infection. Moreover, recent findings suggest that the abundance of T4 isolates in human infections is most likely due to their greater virulence and/or properties that enhance their transmissibility as well as their decreased susceptibility to chemotherapeutic agents (Visvesvara et al. 2007). *Acanthamoeba* are found in diverse habitats. This is not surprising, since we often come across and interact with these organisms. More than 80 % of the normal human population exhibited antibodies against *Acanthamoeba* (Chappell et al. 2001), suggesting that these organisms often come into contact with humans. It is noteworthy that during this investigation, we have isolated T4 genotype from different water resources including household drinking water, air conditioning unit, and sewage water samples at a rate of 4 out of 10 (40 %). However, the presence of only a limited number of genotypes T4 in our study is somewhat surprising, as it has been reported previously that T4 is a highly prevalent genotype in environment (Niyyati et al. 2009). In Karachi, Pakistan, the first report of *Acanthamoeba* keratitis human infection (identified from a clinical sample for the first time) dates back to the year 2001 (Gast 2001). In addition, *Acanthamoeba* cysts were recovered from human feces in Karachi, Pakistan (Zaman et al. 1999), suggesting the presence of *Acanthamoeba* species in Pakistan, but this could be due to imported cases.

This is not a novel concept, as the genotypes identified during this study have also been isolated previously from variety of environments from different parts of the world, but the interesting findings of the present study was identification of novel genotype T17, which was reported recently from Thailand (Nuprasert et al. 2010). Although 92 % of collected samples demonstrated the outgrowth of *Acanthamoeba* on non-nutrient agar plates, which resembled morphologically the amoebic cysts, when these samples (amoeba cysts) were PCR amplified using *Acanthamoeba* genus specific primers, only ten of them could be confirmed as *Acanthamoeba*, which may be due to many reasons. It is reasonable to predict that the non-amplified products point towards the presence of other protozoan cyst, which needs further investigation, because one of the previous studies (Arnalich-Montiel et

al. 2012) has shown the co-isolation of two different amoebae (*Vahlkampfia* and *Acanthamoeba*) from Spain. This will be the possible future potential questions to be address for their molecular identification.

Conclusions

To the best of our knowledge, this will be the first study which demonstrates the presence of *Acanthamoeba* in the water sources in KPK, Pakistan. We identified pathogenic and nonpathogenic *Acanthamoeba* genotypes in different water resources (drinking, air conditioning, canal, and sewage water) of Pakistan, which is quite frightening from public health perspective. Serious actions should be taken to avoid any epidemic in the future, like *Acanthamoeba* keratitis (Schaumberg et al. 1998). Early diagnosis followed by aggressive treatment is essential for the successful prognosis. Hence, awareness among clinicians would be the key factor which will help in proper diagnosis of *Acanthamoeba* infections; ultimately, therapeutic measures could be taken for in time treatment. There is an urgent need to explore other environmental sources in Pakistan for more detailed knowledge about the distribution of *Acanthamoeba* genotypes and their potential threat to human health. Therefore, this report is in continuation of the other series of studies, which are still underway for the analysis of different environmental sources like soil, air, and water samples across Pakistan. Further studies are necessary in order to determine the pathogenic potential of the identified genotypes and their role in the environment. These findings serve as additional evidence for the presence of pathogenic *Acanthamoeba* strains in habitats related directly to human populations, and which could be a risk for human health.

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Conflicts of interest Authors have no conflict of interest to declare.

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