SHORT COMMUNICATION

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Quick survey for detection, identification and characterization of *Acanthamoeba* genotypes from some selected soil and water samples in Pakistan

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Abstract

Acanthamoeba is an opportunistic protozoan pathogen which is widely distributed in nature and plays a pivotal role in the ecosystem. *Acanthamoeba* species may cause blinding keratitis and fatal granulomatous encephalitis involving the central nervous system. This study investigates the presence of *Acanthamoeba* in soil and water resources in Pakistan. *Acanthamoeba* were recovered on non-nutrient agar plate lawn with *E. coli* and identified by morphological characteristics of the cyst. PCR was performed with genus-specific primers, followed by direct sequencing of the PCR product for molecular identification. Overall, the PCR and sequencing results confirmed pathogenic genotypes, including T4 and T15, from both soil and water samples. This is the first report of *Acanthamoeba* isolation from both soil and water resources in Pakistan which may serve as a potential threat to human health across the country.

Key words

Acanthamoeba, genotyping, soil resources, water resources, PCR, Pakistan

INTRODUCTION

Acanthamoeba is free-living protozoan pathogen widely distributed in nature. They could be found in diverse soil, dust, air and water environment [1, 2, 3]. In addition, they have been isolated from vegetables, some animals (fish, reptiles, amphibians, dogs, monkeys and birds), pulmonary secretions, maxillary sinus and stool samples [4], and are known to be one of the most ubiquitous organisms. The Genus *Acanthamoeba* consists of both pathogenic and non-pathogenic isolates. Given the correct access and host conditions, pathogenic *Acanthamoeba* can cause serious human diseases, such as eye keratitis, or the rare and life-threatening *Acanthamoeba* granulomatous encephalitis [1, 2]. Given the free-living nature of the organisms, it is anticipated that humans encounter *Acanthamoeba* during their normal life. The genus *Acanthamoeba* has been classified into 17 different genotypes (T1–T17) and has been isolated from environmental resources worldwide [5]. To the best of our knowledge, this is the first study demonstrating the isolation and molecular characterization of *Acanthamoeba* genotypes in soil as well as water resources in Pakistan.

MATERIALS AND METHOD

Cultures of *Acanthamoeba and Escherichia coli.* A clinical isolate of *Acanthamoeba castellanii* belonging to the T4 genotype isolated from a Keratitis patient (ATCC 50492) was used as a control. *Acanthamoeba* were grown without shaking in 15-ml of PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] in T-75 tissue culture flasks at 30 °C, as previously described [6]. Gram-negative bacteria *E. coli* K12 (HB101), which is a non-invasive laboratory strain, were also used in this study. Bacteria were grown overnight in Luria-Bertini (LB) medium, containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl, as described previously [7].

Sample collection, processing and *Acanthamoeba* isolation on nutrient agar plates. A survey was conducted of *Acanthamoeba* isolation in 20 representative cities of Pakistan. The soil and water samples were collected from different types of resources from January 2012 – December 2012. The total water sample volume of 500 ml was thoroughly mixed and filtrated through a cellulose nitrate filter, as described previously [8]. Each filter was placed upside down on 1.5% non-nutrient agar plates seeded with heat-killed *E. coli* (a non-invasive strain HB101). The plates were then incubated at 30 °C for up to 2 weeks until growth of amoeboid plaques was visible. During this incubation period, amoebic growth was examined daily by inverted phase microscopy. In contrast, soil samples (2 g) were collected and dissolved in 20 ml of distilled sterile water, and 150 µl of each sample was inoculated onto 1.5% non-nutrient agar plates, as described previously [9].

DNA extraction from purified samples obtained from platting assays. Amoeboid plaques were scraped off the agar plates and DNA extraction performed, as described previously [8, 10]. Briefly, the cells were palleted at 10,000g for 5 min at RT, followed by resuspension in lysis buffer

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[(100mM KCl, 40mM Tris, 5mM MgCl2, 1% (w/v) Tween-20 and 100 µg/ml proteinase K)]. Next, the tubes were incubated for 1h at 56°C, followed by 10min incubation at 100°C to inactivate proteinase K. Finally, the tubes were centrifuged at 10,000g for 5min and supernatants collected and used as DNA template. The purity of DNA was determined by using a Hitachi U-3210 spectrophotomer.

Identification of *Acanthamoeba* using PCR amplifications and genotyping. The DNA amplification reactions were performed using the genus-specific primers, as described previously [8, 10]. Primer pair includes the forward primer JDP1 (5-GGCCCAGATCGTTTACCGTGAA) and the reverse primer JDP2 (5-TCTCACAAGCTGCTAGGGAGTCA). PCR reaction was performed in 20µl volume containing 1.25 U Taq (Qiagen), 0.2–0.4µg DNA, 200µM dNTPs, 2 mM $MgCl_2$ and 2µM primer at 95°C for 5 min for 1 cycle, 94°C for 1min, 60°C for 1min and 72°C for 2 min for 35 cycles, and a final elongation step of 10min at 72°C. Amplified DNA was electrophoresed on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet (UV) light. After purification, direct sequencing of 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 [11].

Accession numbers. Sequences are deposited in the GenBank, Acession Nos. KC778186 – KC778190 and KF035055 – KF035057.

RESULTS

Overall, 16 of the 17 soil (94.11%) and 12 of the 13 water samples (92.31%) were positive for the outgrowth of amoeba on non-nutrient agar plates. In total, 28 of 30 (93.33 %) among them, 10 of 17 (58.82%) soil and 11 of 13 (84.61%) water samples, were successfully amplified using *Acanthamoeba* genus specific primers (in total: 21 of 30 (70 %). Alternately, 4 of the 17 (23.53%) soil and 4 of the 13 (30.77%) water cultures containing *Acanthamoeba* were genotyped based on the DF3 sequence. Results revealed that the isolated *Acanthamoeba* strains belonged to T4 (84.6%) and T15 (7.6%) genotypes (Tab. 1).

Several studies have shown *Acanthamoeba* isolation from soil and water resources worldwide related to human habitats (5, 12); but despite the free-living nature of *Acanthamoeba* and its presence in various environments, to-date, there is not a single reported case of keratitis or encephalitis infections from Pakistan, which may be attributed due to the lack of expertise and awareness even among the clinician community. Although the authors of the presented study have previously produced an extensive water evaluation survey for the isolation of *Acanthamoeba* from Khyber Pakhtunkhwa (KPK), Pakistan [8], in the presented study they show for the first time pathogenic (T4 and T15) *Acanthamoeba* genotypes isolated from soil resources of Pakistan (Fig. 1).

It is worth noting that during this investigation, the T4 genotype was isolated from different water resources

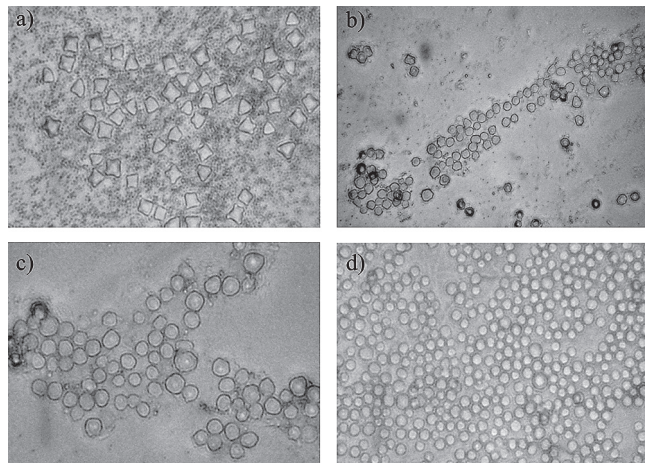

Figure 1. *Acanthamoeba* cysts (×400) on non-nutrient agar plates when observed under inverted microscope. Soil and water samples were pored and filtered, respectively, and inoculated on non-nutrient agar plate lawn with *E. coli,* as described in Materials and methods. Plates were observed for outgrowth up to 14 days and images made. Representative samples (a) KHI-TW4 (b) IBD-TW1 (c) LHR-FS3 (d) KHI-SS2 are shown

in 4 out of 10 (40%) samples, which suggests a very low prevalence of *Acanthamoeba* T4 genotype in water resources in Pakistan. However, the presence of only a limited number of *Acanthamoeba* genotypes in the presented study from selected cities across the country is not surprising. The authors have also shown recently the tribal population of KPK has a high titer level of anti-*Acanthamoeba* antibodies [13], compared with the rest of the country, which supports the finding that *Acanthamoeba* genotypes are very limited in other parts of the country, except KPK. Overall, the majority of soil and water samples were amplified with *Acanthamoeba* genus-specific primers, but conclusive sequences could not be obtained and still need to be further investigated. It is speculated that the amplified samples might be the a novel species (Fig. 2), which will be addressed in future studies by the authors. Therefore, the information provided in this study may serve as a base-line for future studies on the role of free-living amoebae, e.g. in outbreaks of water- and soil borne-diseases in the country.

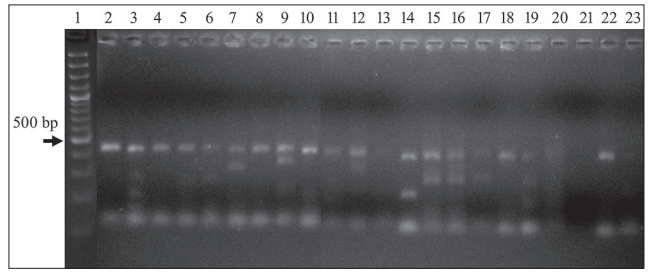

Figure 2. PCR analysis of the amoeba isolated from soil and water samples from representative cities of Pakistan. To confirm the presence of *Acanthamoeba* populations, DNA were isolated from amoeba recovered from non-nutrient agar plates after 7–14 days and used for PCR analysis using *Acanthamoeba*-genus specific primers pairs JDP1 and JDP2, as described in Materials and Methods. PCR products were found to be about 500 bp in all samples, confirming the presence of *Acanthamoeba*. Lane 1: 100 bp DNA ladder; Lane 2: +ve control; Lane 3: IBD-TW1; Lane 4: IBD-FS2; Lane 5: KHI-TW4; Lane 6: CHAR-FS4; Lane 7: KHI-SS2; Lane 8: LHR-FS1; Lane 9: LHR-FS3; Lane 10: KHP-TW1; Lane 11: LKA-TW7; Lane 12: MLT-RBS1; Lane 13: LKA-SS7; Lane 14: PSH-BW3; Lane 15: PSH-CW6; Lane 16: PSH-FS9; Lane 17: MUZ-FS2; Lane 18: SRA-TW6; Lane 19: SKD-FS2; Lane 20: SIBI-TW6; Lane 21: WAGA-DS2; Lane 22: QTA-TW3; Lane 23: -ve control

Table 1. *Acanthamoeba* genotypes identified in different soil and water resources from representative cities of Pakistan

	Sample code	Sources	**S**ampling area	DNA tube	NNA Culture	PCR	Species	Genotypes	GenBank Accession No
1	BANU-FS1	Fertile soil	Bannu	81	+	-	ND	-	-
2	CHAR-FS4	Fertile soil	Charsadda	84	+	+	ND	-	-
3	DADDO-SS6	Sandy soil	Daadoo	67	+	-	ND	-	-
4	DIK-DS1	Deserted soil	Dera Ismail Khan	74	+	+	ND	-	-
5	IBD-TW1	Tap water	Islamabad	94	+	+	*Acanthamoeba jacobsi*	T15	KC778190
6	IBD-FS2	Fertile soil	Islamabad	95	+	+	*Acanthamoeba* sp	-	KC778188
7	KHI-TW4	Tap water	Karachi	114	+	+	*Acanthamoeba* sp	T4	KC778187
8	KHI-SS2	Sandy soil	Port Qasim	103	+	+	*Acanthamoeba jacobsi*	T15	KC778189
9	KA-DS1	Deserted soil	Khyber Agency	86	+	-	ND	-	-
10	KHP-TW1	Tap water	Khaplu	111	+	+	ND	-	-
11	LHR-FS1	Fertile soil	Lahore	14	+	+	*Acanthamoeba* sp	T4	KC778186
12	LHR-FS3	Fertile soil	Lahore	25	+	+	*Acanthamoeba* sp. 1 AM-2013	T4	KF035056
13	LHR-TW4	Tap water	Lahore	3	+	+	ND	-	-
14	LKA-SS7	Sandy soil	Larkana	75	-	-	-	-	-
15	LKA-TW7	Tap water	Larkana	63	+	+	ND	-	-
16	MNI-PS2	Potted Soil	Mianwali	60	+	+	ND	-	-
17	MNI-CW3	Canal water	Mianwali	42	+	+	ND	-	-
18	MLT-CW3	Canal water	Multan	78	+	+	ND	-	-
19	MLT-RBS1	River bank soil	Multan	99	+	+	ND	-	-
20	MUZ-FS2	Fertile soil	Muzafrabad	105	+	-	ND	-	-
21	PSH-BW3	Boring water	Peshawar	104	+	+	*Acanthamoeba jacobsi*	T15	KF035057
22	PSH-CW6	Canal water	Peshawar	15	+	+	*Acanthamoeba* sp. 2 AM-2013	-	KF035055
23	PSH-FS9	Fertile soil	Peshawar	90	+	+	ND	-	-
24	SKD-RW1	River water	Sakardu	150	+	-	ND	-	-
25	SKD-FS2	Fertile soil	Sakardu	132	+	-	ND	-	-
26	SRA-TW6	Tap water	Sargodha	36	+	+	ND	-	-
27	SHI-FS2	Fertile soil	Shinkiari	18	+	+	ND	-	-
28	SIBI-TW6	Tap water	Sibi	214	-	-	-	-	-
29	QTA-TW3	Tap water	Quetta	221	+	+	ND	-	-
30	WAGA-DS2	Deserted soil	Waga	28	+	-	ND	-	-

ND – Not Determined

CONCLUSIONS

To the best of the authors' knowledge, this is the first report which demonstrates the presence of *Acanthamoeba* in the soil and water sources of Pakistan. Pathogenic *Acanthamoeba* genotypes were identified in fertile (decomposed) soil, which is usually preferred for the cultivation of various crops in Pakistan; this is quite frightening from the public health perspective. In addition, pathogenic *Acanthamoeba* species were identified from tap water normally consumed by the human population across the country. This, besides confirming the presence of pathogenic species in both soil and water medium, also indicates that they may also serve as a transmission vehicle of pathogenic bacteria in different environmental sources in Pakistan – an additional potential threat to humans. This report is a continuation of the other series of studies which are still underway for the analysis of different environmental sources, such as soil, air and water samples across the country. Further studies are necessary in order to determine the pathogenic potential of the identified genotypes and their role in the environment.

Authors' contributions. AM and TT surveyed the area, collected and processed the samples for amoeba isolation on NNA plates. AH, AG and TT prepared each sample for further molecular analyses, carried out PCR, genotyping, and participated in the interpretation and analysis of data; AM conceived the study, designed the study protocol and carried out interpretation of data and drafted the manuscript. All authors read and approved the final manuscript. AM is guarantor of the paper.

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Conflicts of interest. The authors have no conflict of interest to declare.

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