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Short communication

Isolation of Thermotolerant Pathogenic *Naegleria australiensis* from Diverse Water Resources Including Household Drinking Water in Pakistan

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Abbreviations

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
FLA	Free-living amoebae
ITS	Internal transcribed spacers
KCl	Magnesium chloride
LB	Luria-Bertini
$MgCl_2$	Magnesium chloride
NNA	Non-nutrient agar
PAM	Primary amoebic meningoencephalitis
PCR	Polymerase chain reaction

Abstract. *Naegleria* is well recognized for primary amoebic meningoencephalitis which always results into death. To date there is not a single report demonstrating molecular identification of *Naegleria* from water resources of Pakistan thus the aim of the proposed study. Here, in total 135 various water samples (like domestic tap water, municipal water, sea water, well water, tube well water, canal water, boring water, sewage water, lake water and stream water) were collected across Pakistan and evaluated for *Naegleria* species. *Naegleria australiensis* (pathogenic) and *Naegleria lovaniensis* (non pathogenic) were isolated on non nutrient agar plates and were further identified by PCR and sequencing. To best of our knowledge we have described for the first time the isolation and molecular identification of thermotolerant pathogenic and non pathogenic *Naegleria* species from diverse water samples including drinking water across Pakistan. The presence of pathogenic *Naegleria* species in diverse water samples may add the health threat to the community.

Key words: Thermotolerant pathogenic *Naegleria*, Primary Amoebic Meningoencephalitis, Isolation, Molecular identification, Pakistan

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INTRODUCTION

Free-living amoebae (FLA) are highly diverse and show a worldwide distribution in various environments (Jamerson *et al*. 2009; Behets *et al*. 2007; Marciano-Cabral and Cabral 2007; Gianinazzi *et al*. 2009). Among FLA, *Naegleria fowleri* is well known to be the cause of primary amoebic meningoencephalitis in humans. More than 30 species of *Naegleria* have been reported from diverse environment and *Naegleria fowleri* for many years was the only species of this genus known to infect human. However, *Naegleria australiensis* is another pathogenic species of *Naegleria* which were identified as cause of PAM infections in Australia in 1965. *Naegleria* species have been isolated from natural and man-made aquatic habitats and even from normal drinking water (Schuster and Visvesvara 2004). It is generally acquired while swimming, diving in freshwater lakes and ponds (Martinez and Visvesvara 1997). The current study was planned after thirteen cases of *Naegleria fowleri* PAM in human were reported in a period of just 17 months in Karachi, Pakistan (Shakoor *et al*. 2011). We therefore undertook the following comprehensive survey to investigate the molecular identification of *Naegleria* in various water resources across Pakistan in order to evaluate the future possible risk for human health in the country.

MATERIALS AND METHODS

Collection, processing & cultivation of water samples on non nutrient agar plates

The water samples were collected across Pakistan from January 2012 to December 2012. In total 135 water samples (500 ml in volume each) from diverse sources like domestic tap water, , sea water, well water, tube well water, canal water, boring water, sewage water, lake water and stream water) were collected across Pakistan. Samples were collected and stored in sterilized polypropylene bottles labelled with date, time and place of collection. For *Naegleria* isolation plating assay was performed by modifying previous protocols (Ithoi *et al*. 2011). Briefly bacteria were grown in LB for overnight and were heat-killed as described previously (Matin and Jeong 2011). Heat-killed bacteria were poured on to non-nutrient agar plates and left for 2–3 minutes. The excess cultures were poured off and plates left to dry. Water samples (500 ml) were filtrated through a nitrocellulose membrane (pore size: 0.2 µm) and filters were inserted upside down on non-nutrient agar plates seeded with *E. coli* and plates were incubated at 30, 42 and 45°C (Fig. 1a). Plates were kept sealed in plastic bags to prevent drying and examined with an inverted microscope. Following, the detection of amoebas feeding on *E. coli* on agar, portions of the agar that contained these amoebas were excised and transferred onto newly bacteria-coated plates. After the amoebas of interest had migrated away from fungal and other contaminants, they were then transferred in agar cores to fresh, bacteria-coated agar plates. The agar plates were monitored for out-growth of *Naegleria* (trophozoites or cysts) microscopically for up to 2 weeks (Fig. 1b).

DNA extraction from amoebic plaque obtained from non nutrient agar plates

Amoeboid plaques were scraped off from the agar plates and DNA extraction was performed as described previously (Edagawa *et al*. 2009). Briefly, the cells were palleted at 10,000g for 5 min at RT followed by re-suspension in lysis buffer [(100 mM KCl, 40 mM Tris, 5 mM $MgCl_2$, 1% (w/v) Tween-20 and 100 µg/ml proteinase K)]. Next, tubes were incubated for 1h at 56°C followed by 10 min incubation at 100°C to inactivate proteinase K. Finally, the tubes were centrifuged at 10,000g for 5 min and supernatants were collected and used as DNA template.

Polymerase chain reaction analysis and sequencing

Polymerase chain reaction (PCR) was performed using ribosomal internal transcribed spacers (ITS) primers by modifying previous protocols (Sheehan *et al*. 2003). Briefly, 25 µl of PCR mixture contained 2 µl of the extracted DNA, 2.5 µl of 10 × PCR buffer (100 mM KCL, 20 mM $MgCl_2$, 20 mM Tris–HCl [pH 8.0]), 1.5 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTP mixture, 0.75 µl of each 100 µM primer, and 0.25 µl of 5 U/µl Ex Taq DNA polymerase. PCR-amplification was performed with a genus specific primer set ITS1_F and ITS1_R. Reaction conditions were 95°C for 5 min followed by 30 cycles of 95°C for 15 s, 53°C for 1 min 30 s, 72°C for 1 min 30 s, and extension at 72°C for 10 min. PCR products were electrophoresed on a 2% agarose gel and stained with a solution of 0.5 µg/ml of ethidium bromide and visualized under UV light. PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using a sequencing kit (Applied Biosystems, Foster City, USA). A homology search was performed with BLAST software from the NCBI.

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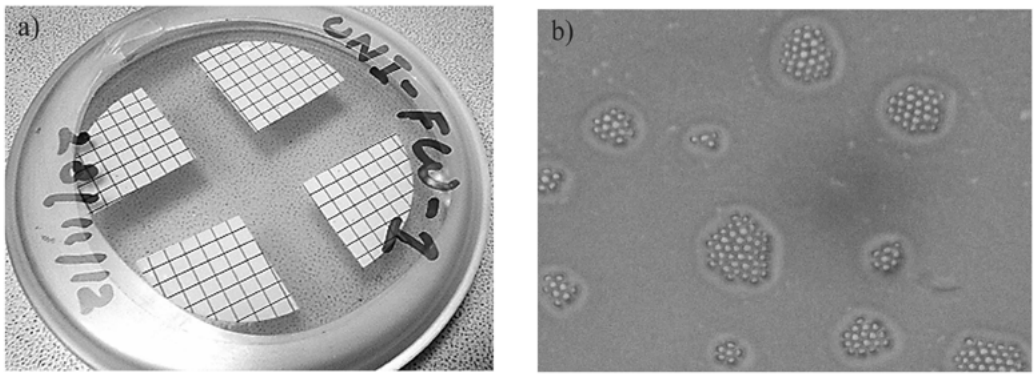
Fig. 1. (a) Water sample (500 ml) was thoroughly mixed and filtrated through a cellulose nitrate filter and inoculated on non-nutrient agar (NNA) plate lawn with *E. coli* as described in Materials and Methods. **(b)** Amoebic plaque cysts (× 400) were morphologically observed up to 14 days on NNA plates under inverted microscope and images were taken. **(c)** Neighbour joining phylogenetic relationship between the partial sequences of 18S rRNA of *Naegleria* from isolates obtained in this study and reference sequences present in Gen Bank. The tree was generated in CLC Main Workbench version 6.6.2 using 1000 bootstrap replications. Branch length is proportional to the calculated genetic distance (scale shown).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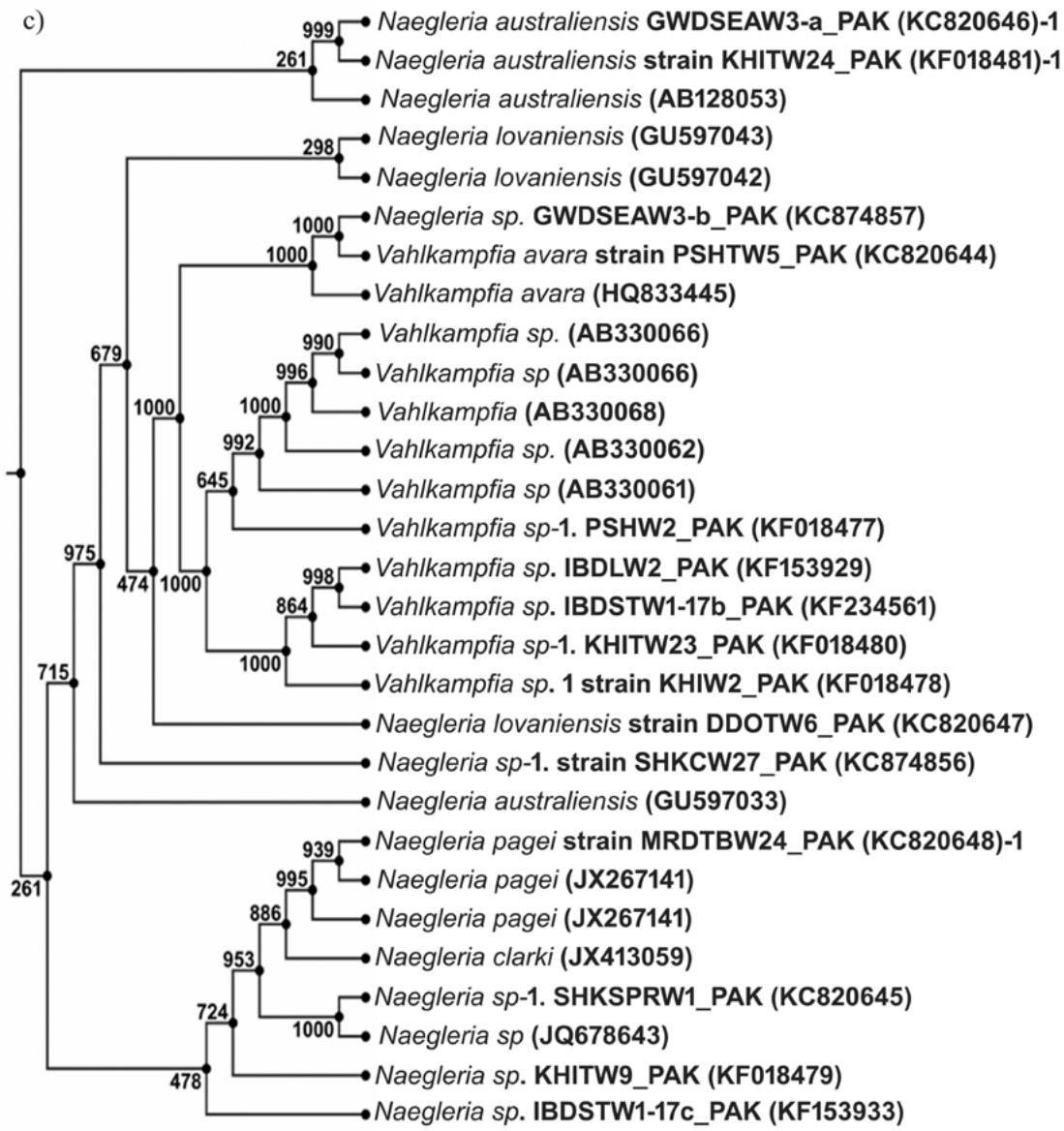
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886
953
724
1000
478
Naegleria australiensis **GWDSEAW3-a_PAK (KC820646)-1**
Naegleria australiensis **strain KHITW24_PAK (KF018481)-1**
Naegleria australiensis **(AB128053)**
Naegleria lovaniensis **(GU597043)**
Naegleria lovaniensis **(GU597042)**
Naegleria sp. **GWDSEAW3-b_PAK (KC874857)**
Vahlkampfia avara **strain PSHTW5_PAK (KC820644)**
Vahlkampfia avara **(HQ833445)**
Vahlkampfia sp. **(AB330066)**
Vahlkampfia sp **(AB330066)**
Vahlkampfia **(AB330068)**
Vahlkampfia sp. **(AB330062)**
Vahlkampfia sp **(AB330061)**
Vahlkampfia sp-**1. PSHW2_PAK (KF018477)**
Vahlkampfia sp. **IBDLW2_PAK (KF153929)**
Vahlkampfia sp. **IBDSTW1-17b_PAK (KF234561)**
Vahlkampfia sp-**1. KHITW23_PAK (KF018480)**
Vahlkampfia sp. **1 strain KHIW2_PAK (KF018478)**
Naegleria lovaniensis **strain DDOTW6_PAK (KC820647)**
Naegleria sp-**1. strain SHKCW27_PAK (KC874856)**
Naegleria australiensis **(GU597033)**
Naegleria pagei **strain MRDTBW24_PAK (KC820648)-1**
Naegleria pagei **(JX267141)**
Naegleria pagei **(JX267141)**
Naegleria clarki **(JX413059)**
Naegleria sp-**1. SHKSPRW1_PAK (KC820645)**
Naegleria sp **(JQ678643)**
Naegleria sp. **KHITW9_PAK (KF018479)**
Naegleria sp. **IBDSTW1-17c_PAK (KF153933)**

Phylogenetic analysis

Multiple alignment was performed using Clustal Omega (Larkin *et al*. 2007) in comparison with sequences available in GenBank for *Naegleria*. Phylogenetic reconstruction produced a gene tree by using neighbor-joining in the phylogenetic computer program (CLC main work bench 6.6.2) as described previously (Tamura *et al*. 2011).

Accession numbers

Sequences were deposited in Gen Bank with accession numbers KC820644–KC820648, KC874856, KC874857, KF018477–KF018481, KF153929, KF153933 and KF234561.

RESULTS AND DISCUSSION

Overall, 96 out of 135 (71.11%) water samples were positive for amoebae outgrowth on non nutrient agar plates at 30°C while 2 (2.22%) at 42°C and 2 (2.22%) at 45°C. Cultures containing amoeba like outgrowth were further confirmed as *Naegleria* sp. after PCR and sequencing. Results revealed that 34 (35.41%) samples were positive for *Naegleria* DNA which were further identified *Naegleria* sp. 09 (26.47%) and *Vahlkampfia* sp. 06 (17.64%) (Table 1). We successfully recovered *Naegleria* species on NNA plates at 30, 42 and 45°C. Interestingly, two *Naegleria* species were identified from a single water sample (GWD-SEAW3). It is noteworthy that in some of samples, *Acanthamoeba* was also isolated along with *Naegleria* species from the same water sample (data not shown). In support our group has recently isolated *Acanthamoeba* species from water resources of Khyber Pakhtunkhwa, Pakistan (Tanveer *et al*. 2015; Tanveer *et al*. 2013). In this regard further studies are necessary to evaluate the properties and quality of water which support amoebae species and *Naegleria* in particular. To the best of our knowledge, this is the first report demonstrating the exploration of various water resources and isolation and identification of pathogenic *Naegleria* species across Pakistan.

It is noteworthy findings of the present study that two thermotolerant species i.e. *Naegleria australiensis* and *Naegleria lovaniensis* were successfully recovered and identified. Like *Naegleria fowleri*, these identified *Naegleria* species tolerate temperatures up to 45°C and propagate tremendously during summer in natural water resources like lakes, rivers, geo-thermally heated water and industrial cooling water. These thermotolerant species are competitors that develop better than *Naegleria fowleri* at these temperatures. Furthermore it is well established that FLA also act as a host for several bacteria, and its ability to host bacterial pathogens has gained particular attention in recent years. These bacterial pathogens not only survive intracellularly but also multiply within them. This allows bacteria to transmit throughout the environment, evade host defences and/or chemotherapeutic drugs, and reproduce in sufficient numbers to produce disease. Upon favorable conditions, the increasing bacterial densities lyse their host amoebae and infect new amoebae and/or produce disease. This ability of FLA to resist harsh conditions (such as osmolarity, pH and extreme temperatures), especially during their cyst stage, suggest their usefulness as bacterial vectors. In particular, FLA cysts are notoriously resistant to chlorine. This poses clear challenges in eradicating bacterial pathogens from public water supplies, especially in developing countries (Rowbotham 1986; Michel *et al*. 2000). Furthermore, amoeba-bacteria interactions also effect bacterial virulence; i.e. pneumophila grown within amoeba exhibited increased motility, drug resistance and virulence compared with axenically grown *Legionella* (Greub and Raoult 2004). Prevalence of pathogenic bacteria inside drinking water reservoirs and their symbiotic relationship with *Naegleria* could be a potential question to be addressed in future.

According to the reports worldwide single specie *Naegleria fowleri* is responsible for PAM but none of the isolate could be identified as *Naegleria fowleri* during this study. However, the widespread presence of thermotolerant *Naegleria* in water supplies, especially *Naegleria lovanensis*, which is an indicator species for *Naegleria fowleri*, suggests this pathogenic amoeba may pose a risk to public health in the area (Marciano-Cabral and Cabral 2007). In support at least 13 PAM cases have already been reported in Karachi, Pakistan, who had no history of aquatic activities and it was believed the infections likely occurred through ablution with tap water (Shakoor *et al*. 2011). There are few reports of PAM caused by Naegleria have been reported from Pakistan previously (Saleem *et al*. 2009; Shariq *et al*. 2014). Seven out of 15 (46.66%) *Naegleria* species have been identified during this study but originally 15 out of 59 (25.42%) samples were positive for *Naegleria* DNA during PCR. Overall, the majority of water samples were amplified with *Naegleria* 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 novel species, which will be addressed in future studies. Therefore, the information presented in this report

Table 1. Naegleria species identified from diverse water resources across Pakistan.

	Samples codes	Source	Sampling area	NNA culture (°C)			PCR	*Naegleria* species identified	Gene Bank accession no	Homology %
				30	42	45				
1	BAN-TW1	Tap water	Bannu	+	–	–	+	ND	ND	ND
2	DDO-TW6	Tap water	Daadoo	+	–	–	+	*Naegleria lovaniensis*	KC820647	98%
3	DDO-WW6	Well water	Daadoo	+	–	–	+	ND	ND	ND
4	DIK-W1	Boring water	D-I-Khan	+	–	–	+	ND	ND	ND
5	GWD-CW3	Canal water	Gawadar	+	–	–	+	ND	ND	ND
6	GWD-SEAW3	Sea Water	Gawadar	+	–	+	+	*Naegleria* sp. AM-2013a	KC874857	84%
	GWD-SEAW3	Sea water	Gawadar	+	+	–	+	*Naegleria australiensis*	KC820646	98%
7	HYD-W2	River water	Hyderabad	+	–	–	+	ND	ND	ND
8	IBD-STW1	Stagnant water	Islamabad	+	–	–	+	*Vahlkampfia* sp. 1 AM-2013	KF234561	94%
9	IBD-STW2	Stagnant water	Islamabad	+	–	–	+	*Naegleria* sp.	KF153933	99%
10	IBD-LW3	Lake water	Islamabad	+	–	–	+	*Vahlkampfia* sp.	KF153929	98%
11	RWP-TW8	Tap water	Rawalpindi	+	–	–	+	ND	ND	ND
12	GWA-MW4	Municipal water	Gujrawala	+	–	–	+	ND	ND	ND
13	LHR-TW7	Tap water	Lahore	+	–	–	+	ND	ND	ND
14	LHR-MW5	Municipal water	Lahore	+	–	–	+	ND	ND	ND
15	KHI-TW9	Tap water	Karachi	+	–	–	+	*Naegleria* sp. 1 AM-2013	KF018479	94%
16	KHI-TW23	Tap water	Karachi	+	–	–	+	*Vahlkampfia* sp.	KF018480	97%
17	KHI-TW24	Tap water	Karachi	+	+	–	+	*Naegleria australiensis*	KF018481	99%
18	KHI-W2	Boring water	Karachi	+	–	–	+	*Vahlkampfia* sp. 1 AM-2013	KF018478	86%
19	KHI-SW3	Sewage water	Karachi	+	–	–	+	ND	ND	ND
20	KF-TW1	Tap water	Khajuri Fort	+	–	–	+	ND	ND	ND
21	LKM-W23	Boring water	Lakimarwat	+	–	–	+	ND	ND	ND
22	MALK-W22	Boring water	Malakand	+	–	–	+	ND	ND	ND
23	MIW-TW12	Tap water	Mianwali	+	–	–	+	ND	ND	ND
24	MRD-TBW24	Tube well water	Mardan	+	–	–	+	*Naegleria pagei*	KC820648	97%
25	PSH-TW5	Tap water	Peshawar	+	–	–	+	*Vahlkampfia avara*	KC820644	99%
26	PSH-W2	Boring water	Peshawar	+	–	–	+	*Vahlkampfia* sp.	KF018477	99%
27	RAW LW2	Lake water	Rawalakot	+	–	–	+	ND	ND	ND
28	RAW TW6	Tap water	Rawalakot	+	–	–	+	ND	ND	ND
29	SHG-TW1	Tap water	Shangar	+	–	–	+	ND	ND	ND
30	SHK-CW27	Canal water	Shinkiari	+	–	+	+	*Naegleria* sp. AM-2013b	KC874856	91%
31	SHK-SPRW1	Spring water	Shinkiari	+	–	–	+	*Naegleria* sp.	KC820645	94%
32	SKD-RW1	River water	Sakardu	+	–	–	+	ND	ND	ND
33	SKD-SW1	Stream water	Sakardu	+	–	–	+	ND	ND	ND
34	TB-W5	Boring water	Tabathar	+	–	–	+	ND	ND	ND

ND: Not determined

may serve as a base-line for further studies on the role of FLA in our environment especially in outbreaks of water borne diseases in Pakistan.

CONCLUSIONS

In conclusion, this study revealed for the first time isolation of thermotolerant both pathogenic and non pathogenic *Naegleria* species from various water samples including the drinking water in Pakistan. It should be stressed that the existence of pathogenic *N. australiensis* in diverse water sources across Pakistan may add the health threat to people of the community. The present findings also encouraged us to reassess these samples and further evaluation of various other environmental sources (i.e., air, soil and water) to know the potential pathogenic amoebic species in the environment which will be helpful for health professionals to adopt strategy for future outbreak caused by *Naegleria* in the country.

Hence, awareness among clinicians would be the key which will help in proper diagnosis of Naegleria infections; ultimately, therapeutic measures could be taken for in time treatment. There is an urgent need to explore other environmental sources across Pakistan for more detailed knowledge about the distribution of Naegleria pathogenic species and their potential threat to human health.

In addition, Naegleria–bacteria interactions also affect bacterial virulence. There is a possible danger that Naegleria may be used as breeding ground for different pathogenic bacteria, which could be a potential threat to human health. The author's future plans are to elucidate the Naegleria-bacteria relationship which would also add threat to human community along with Naegleria.

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