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First report of successful *Naegleria* detection from environmental resources of some selected areas of Rawlakot, Azad Jammu and Kashmir, Pakistan

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Abstract: *Naegleria* belongs to the free-living amoeba family and is well-known as a human pathogen. It is recognized as etiological agent of primary amoebic meningoencephalitis involving central nervous system which always leads to death. To date, there is not a single report demonstrating *Naegleria* isolation and identification from environmental sources of Rawlakot, Azad Jammu and Kashmir Pakistan, and thus the aim of this study. *Naegleria* was isolated on non-nutrient agar plates seeded with heat killed *E. coli* and confirmed by morphological properties of the both stages of cyst or trophozoites. Furthermore, PCR was conducted along with direct sequencing of the PCR product for molecular identification. PCR and sequencing data verified the amplification of *Naegleria* sp. (07) and *Vahlkampfia* sp. (01) from both water and soil samples. Interestingly two species were successfully isolated and cultured on both 30 and 45°C. To the best of our knowledge this is the first report demonstrating the *Naegleria* isolation and molecular characterization from environmental sources of Rawlakot, Azad Jammu and Kashmir, Pakistan. The author is anxious for further evaluation of the pathogenic potential of the identified species and explores drinking water across Pakistan to investigate its quality and frequency of FLA, which might be a possible human hazard in future.

Keywords: *Naegleria*, isolation, environmental sources, PCR, Rawlakot, Pakistan

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INTRODUCTION

The members of free-living amoebae (FLA) are distributed worldwide in diverse environments. Among FLA, *Naegleria* is an amoeboflagellate found in freshwater. It feeds on other microorganisms like bacteria. *Naegleria* is a eukaryotic FLA belonging to the phylum Percolozoa. More than 40 different species of *Naegleria* have been identified but *Naegleria fowleri* is reported to cause primary amebic meningoencephalitis (PAM), a rare but rapidly progressing lethal disease of the central nervous system (CNS) that occurs generally in healthy children and young adults previously exposed to contaminated recreational, ecological and/or household water resources (Marciano-Cabral et al., 2003; Marshall et al. 1997; Martinez 1985; Martinez and Visvesvara 1997; Schuster and Visvesvara 2004a). Infection may also occur when water contaminated with amoebae go into the nasal route of a person while diving, swimming and/or splashing water during ablution. *Naegleria* gets access to the brain and death may occur within 7–10 days. *Naegleria fowleri* grow and multiply in warm water sources, especially at 30°C and above. On the other hand, amoeba is also present in various soils which ultimately contaminate water channels through runoff from the soil after rain (Kyle and Noblet 1985; Singh 1975). Even though PAM is a sporadic infection but the number of reported cases has been increased across the globe since 1990 including Karachi, Pakistan (Ghanchi et al. 2017; Shariq et al. 2014; Shakoor et al. 2011; Saleem et al. 2009).

In addition FLA has been shown to be carriers of potentially pathogenic microorganisms (e.g. *Legionella*) and hence presents an extra threat to human health (Greub and Raoult 2004). Although FLA feeds on bacteria and plays a predatory role in ecosystem but also hosts pathogenic bacteria which become more antibiotic resistant after being hosted by FLA. This property of FLA could possibly operate as a means of transportation of pathogenic bacteria (Greub and Raoult 2004) in various ecological sources across Pakistan which could be a potential additional threat to the public in the future. For example there are quite a few reports demonstrating that FLA assists in survival and escalation of pathogenic *Legionellae* in the natural environment which were observed to be linked with periodic incidence of respiratory diseases in human beings (Abu 1996; Newsome et al. 1998). *Naegleria* has been isolated worldwide along with human reported cases but none from Pakistan so far. To date all PAM reported cases are from Karachi, Pakistan (Ghanchi et al. 2017; Shariq et al. 2014; Shakoor et al. 2011; Saleem et al. 2009) but none from the subject area till today. The author hence carried out the present study to access the presence of *Naegleria* in Rawlakot area in order to estimate the health threat for local population.

MATERIALS AND METHODS

Soil and water samples collection

We carried out an inspection for *Naegleria* detection in 15 representative cities of Rawlakot, Azad Jammu and Kashmir, Pakistan (Table 1). Water and soil samples were collected from various sources of the subject area (shown in Figure-1). All water samples were collected in sterile screw-cap polypropylene bottles followed by labeling with time, date and site of collection information. Furthermore, the soil samples were brought together in sterile plastic bags which were sealed and labeled as described above.

Cultivation of bacteria

Escherichia coli K12 bacteria, a noninvasive laboratory strain (HB101) were utilized during the present investigation. *E. coli* were cultured in LB medium containing Luria–Bertini (LB) medium, carrying 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl overnight and then heat killed as demonstrated earlier (Matin and Jung 2011).

MORPHOLOGICAL IDENTIFICATION

Platting assays

For *Naegleria* isolation, plating assays were carried out as demonstrated earlier (Tanveer et al. 2017; Lorenzo-Morales et al. 2005). Briefly, the heat-killed *E. coli* were transferred onto non-nutrient agar plates (NNA) and placed for 2–3 minutes. Surplus bacteria culture media were discharged off and NNA plates put away to dry. Water samples (500ml) were thoroughly mixed and filtered through sterile nitrocellulose membrane (pore size: 0.2μm). The paper filters possibly containing the contaminants in the water samples were positioned upside down on the 1.5% NNA plates already seeded with heat-killed bacteria and plates were incubated at 30 and 45°C up to 7 days until growth of amoeboid plaques was visible. The 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 nourishing on heat-killed

Table 1. *Naegleria* species detection in soil and water resources of Rawlakot, Azad Jammu and Kashmir, Pakistan

S#	Sample code	Sample Sources	Sampling area	Isolation temperature on NNA plates		PCR	Species identified	Gen Bank accession no.
				30°C	45°C			
1	RAW LW1	Banjosa Lake water	Banjosa	+	-	+	ND	ND
2	RAW PW8	Pond water	Kotari	+	-	+	ND	ND
3	RAW STW7	Stagnant water	Jabur	+	-	+	*Naegleria* sp.	KF153930
4	RAW STW4	Stagnant water	Koat	+	+	+	*Naegleria* sp.	KF442971
	RAW STW4	Stagnant water	Koat	+	+	+	*Naegleria* sp.	KF442972
5	RAW DS1	Decomposed soil	Topa	+	+	+	*Naegleria* sp.	ND
	RAW DS1	Decomposed soil	Topa	+	+	+	*Naegleria* sp.	KF153935
	RAW DS1	Decomposed soil	Topa	+	+	+	*Naegleria* sp. AM-2013b	KF234562
6	RAW TW3	Tap water	Navel	+	-	+	ND	ND
7	RAW DS2	Decomposed soil	Toli	+	-	+	ND	ND
8	RAW DS3	Decomposed soil	Jabur	+	-	+	ND	ND
9	RAW DS4	Decomposed soil	Kotari	+	-	+	*Vahlkampfia* sp.	KF153931
10	RAW DS5	Decomposed soil	Navel	+	-	+	ND	ND
11	RAW DS6	Decomposed soil	kanchi kot	+	-	+	ND	ND
12	RAW DS7	Decomposed soil	Khori channa	+	-	+	*Naegleria* sp.	KF153932
13	RAW DS8	Decomposed soil	Drake	+	-	+	ND	ND
14	RAW DS9	Decomposed soil	Nurkot	+	-	+	ND	ND
15	RAW DS10	Decomposed soil	Pakhur	+	-	+	ND	ND

ND: Not determined

bacteria on agar, segments of the agar which hold amoebae were removed and transported onto a fresh bacteria seeded plates. After the amoeba of interest transferred away from unwanted fungal and other contaminants, they were transported in agar cores to fresh bacteria-coated NNA plates. Furthermore, the plates were observed microscopically for up to 2 weeks for the presence of cysts or/and growth of amoebic trophozoites.

On the other hand, soil samples (2g) were suspended in 20ml of sterile distilled water, and 150µl volume of each sample was inoculated onto 1.5% NNA plates seeded with heat-killed *E. coli* as described above.

MOLECULAR IDENTIFICATION

DNA extraction

Amoeboid plaques were scraped off from the NNA plates and DNA extraction was carried out as described earlier (Tanveer et al. 2017; Edagawa et al. 2009). Briefly, amoebae were rinsed 3× in phosphate buffer saline (PBS) (pH 7.4), centrifuged at 10,000g for 5 minutes at room temperature followed by resuspension in 500µl of cell lysis buffer (100mM KCl, 40mM Tris, 25mM MgCl2, 1% Tween-20, and 0.1mg/ml proteinase K). After gentle mixing by inversion, the lysates were incubated at 56°C for 3h. The tubes were incubated for 1h at 56°C followed by 10min incubation at 100°C to inactivate proteinase K. All samples were chilled on ice for 5 minutes 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 15min at room temperature. 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 precipitated by centrifugation at 15,000 rpm, after washing with 100µl of 70% ethanol, then dried at room temperature, dissolved in 300µl of double-distilled water, and stored at −20°C until used.

PCR amplifications and sequencing

Eppendorf tubes were centrifuged at 10,000g for 5min followed by supernatants collection and used as a DNA template. PCR was carried out using ribosomal internal transcribed spacers (ITS) primers as described previously (Tanveer et al. 2017; Sheehan et al. 2003). Briefly, 25µl of PCR mixture was prepared containing 2µl of the extracted DNA, 2.5µl of 10× PCR buffer (100mM KCL, 20mM MgCl2, 20mM Tris–HCl [pH 8.0]), 1.5µl of 25mM MgCl2, 2µl of 2.5mM dNTP mixture, 0.75µl of each 100µM primer, and 0.25µl of 5 U/µl Ex Taq DNA polymerase. PCR-amplification was carried out with a genus specific primer set ITS1_F and ITS1_R. Reaction conditions were 95ºC for 5 min followed by 30 cycles of 95ºC for 15s, 53ºC for 1min 30s, 72ºC for 1min 30s, and extension at 72ºC for 10 min. PCR products were electrophoresed on a 2% agarose gel followed by a staining with ethidium bromide solution (0.5µg/ml) and visualized under UV light. Next, PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using sequencing kit (Applied Biosystems, Foster City, USA). A homology search was achieved with BLAST software from the NCBI.

Phylogenetic analysis

Multiple alignments were carried out using Clustal Omega as described previously (Larkin et al. 2007) in comparison with sequences already available in GenBank for *Naegleria*. Phylogenetic reconstruction constructed a gene tree by using neighbor-joining in the phylogenetic computer program (CLC main work bench 6.6.2) as demonstrated before (Tamura et al. 2011). Reliability of phylogenetic tree was assessed by bootstrapping phylogenetic reconstruction of 1000 replicates.

Accession numbers

Partial sequences achieved from the present study was submitted in the GenBank database under accession numbers KF153930, KF153932, KF153934, KF153935, KF234562, KF442971 and KF442972 (see Table 1).

RESULTS AND DISCUSSION

PAM causes by *Naegleria* is not a new disease in the recent time. It has been reported back in 1899. Since then hundreds of such cases have been reported all over the world. But in Pakistan first PAM case was reported in 2009 (Saleem et al. 2009), but gained remarkable Pakistani media attention in 2011 when 13 cases were reported within two years in Karachi (Shakoor et al. 2011). PAM is a waterborne infection which is commonly associated with aquatic activities but it can also be transmitted via the domestic water supplies. There are an increased number of PAM cases in Pakistan during this decade (Ghanchi et al. 2017; Shariq et al. 2014; Shakoor et al. 2011; Saleem et al. 2009) but not a single report is available in literature showing the presence of *Naegleria* or demonstration of any proof of isolation or identification of *Naegleria* from any of the environmental sources of Pakistan. In this context, our collaborative research group (members from Oman, Pakistan, Saudi Arabia and South Korea) is the only one which has been working on isolation & molecular characterization of FLA from environmental sources (air, soil and water) of Pakistan since 2010 and shown some novel and interested findings (Tanveer et al. 2017, 2015, 2013). Our group previously demonstrated the identification of thermotolerant pathogenic species of *Naegleria* from various water sources of Pakistan which includes domestic drinking tap in particular and boring water, canal water, lake water, municipal water, sea water, tube well water, well water, river water, stream water, stagnant water even sewage water suggesting the wide distribution of this amoeba in our diverse environment (Tanveer et al. 2017).

Consequently, the present investigation is an attempt in conjunction with other ongoing studies for the examination of diverse environmental resources like air, soil and water media throughout Pakistan. During the present investigation overall, 59 samples (29 soil and 30 water) were evaluated. Forty three (72.88%) samples were cultured positive containing amoeba like growth on non nutrient agar plates. This was further confirmed *Naegleria* DNA after PCR 15 (34.88%) (Fig. 1). This study further revealed seven *Naegleria* sp. (53.33%) and one *Vahlkampfia* sp. (6.66%) from both soil and water samples (Table 1).

It is noteworthy that three different *Naegleria* species were isolated from a single soil (RAW DSL1) and two from single water sample (RAW STW4). Interestingly single soil or water sample produced more than one amplified product on PCR gel suggesting more than one amoebae species was present in each sample. In this regard among water samples RAW PW8, RAW STW4 and RAW TW3 demonstrated two amplified products each. On the other hand soil samples RAW DS1 and

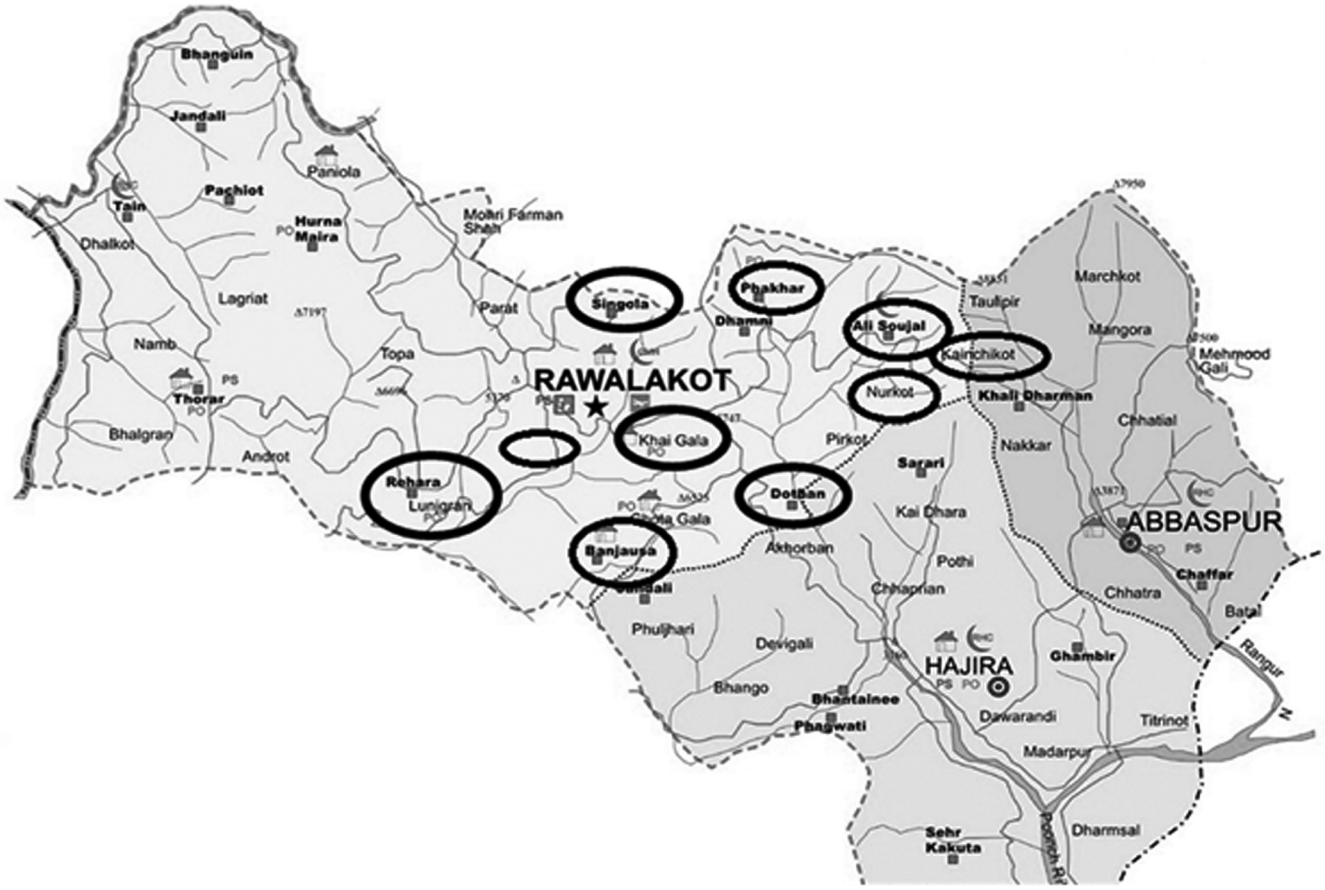


Fig. 1. Sampling sites in the targeted area of Rawlakot, Azad Jammu and Kashmir, Pakistan

RAW DS3 showed three while RAW DS6 and RAW DS9 presented two respectively. Furthermore, amoeba obtained from RAW STW4 and RAW DS1 samples were successfully recovered and cultured on both 30 and 45°C.

PAM infection due to *Naegleria* in Pakistan is not somewhat surprising as at least 13 cases have been identified within 17 months in Karachi only, Pakistan. The victims had no history of aquatic activities and it was assumed these infections were more likely happened during ablution of tap water (Shakoor et al. 2011) particularly among Muslim community. In this context, authors have already detected and indentified various *Naegleria* species from various water and soil resources across Pakistan (Tanveer et al. 2017). In this regard future investigations are essential to examine the characteristics and quality of soil and water sources which assist *Naegleria* species. Rawlakot (33°51'32.18"N, 73° 45'34.93"E) is a town in Azad Jammu and Kashmir, Pakistan which is a wonderful valley surrounded by hills and is the major tourism attractions including mountain-based adventure tourism, rock climbing, mountaineering, trekking, summer camping & hiking, water-based adventure tourism and paragliding. Of note, three main rivers Jhelum, Neelum and Poonch which flows through AJK, to fulfill the water needs across Pakistan.

It is not somewhat surprising that *Naegleria* has been isolated from decomposed soil (which usually enrich with nutrients and microbial flora) on which amoebae feeds on. It has been reported previously that soil is the ultimate habitat for FLA and that runoff water from rains introduced them into water channels (Kyle and Noblet 1985; 1986; 1987; Maclean et al. 2004). Major weather events such as heavy rains and winds were more important than temperature in determining the relative abundance of FLA in aquatic ecosystems (Kyle and Noblet 1985; 1986; 1987). Therefore, it will

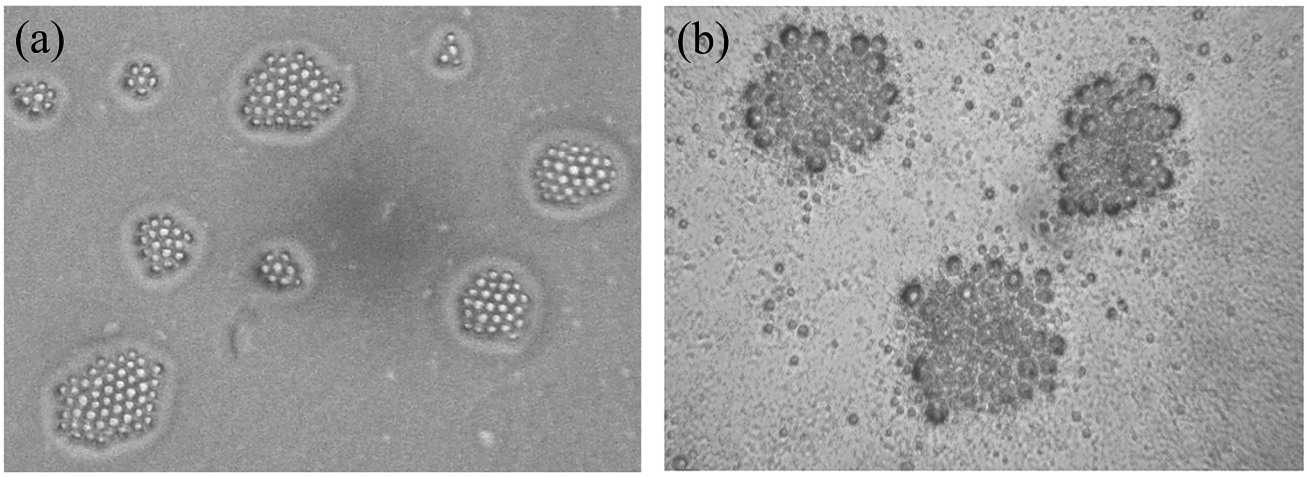


Fig. 2. *Naegleria* cysts detection on NNA under inverted microscope (×400). Water and soil samples were filtered and pored respectively and inoculated on NNA plate seeded with *E. coli* as demonstrated in methods section. Plates were monitored for amoebic outgrowth up to two weeks, and images were taken. Only representative samples of water (a) RAW STW7 and soil (b) RAW DS7 are shown here.

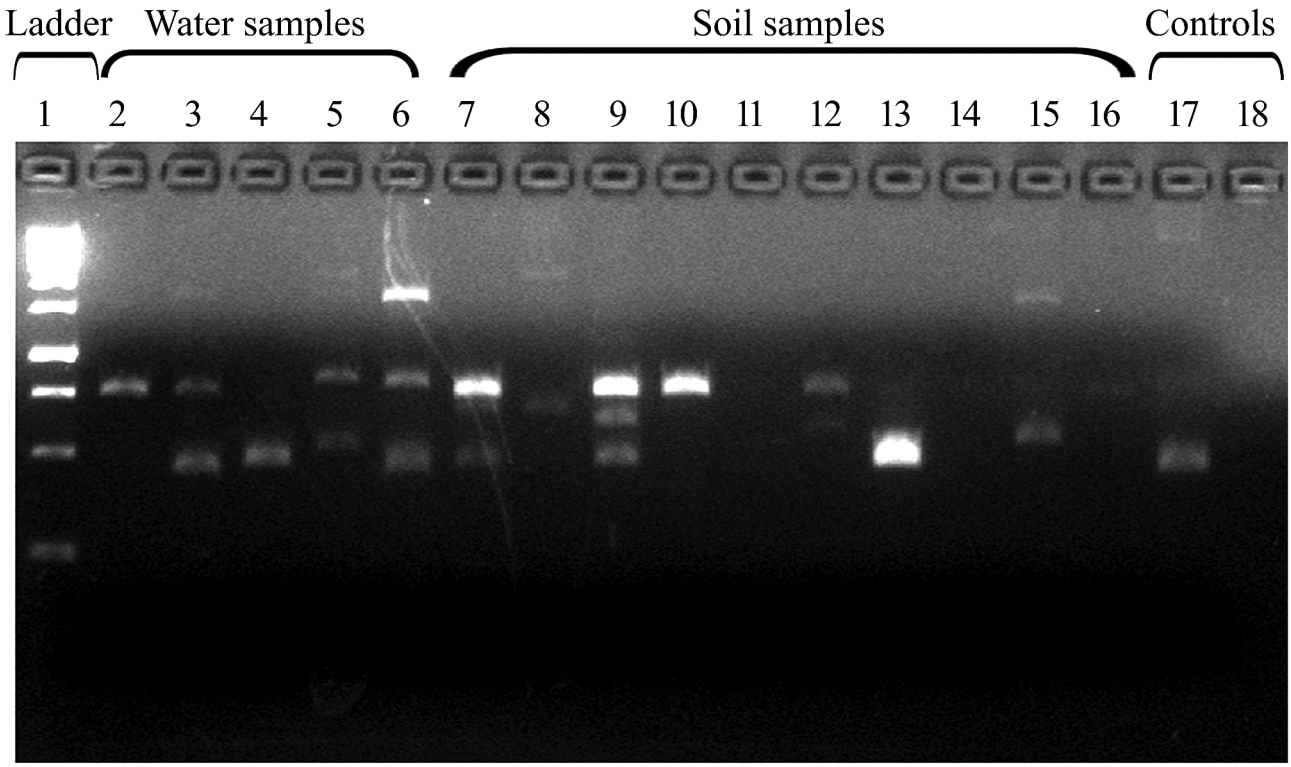
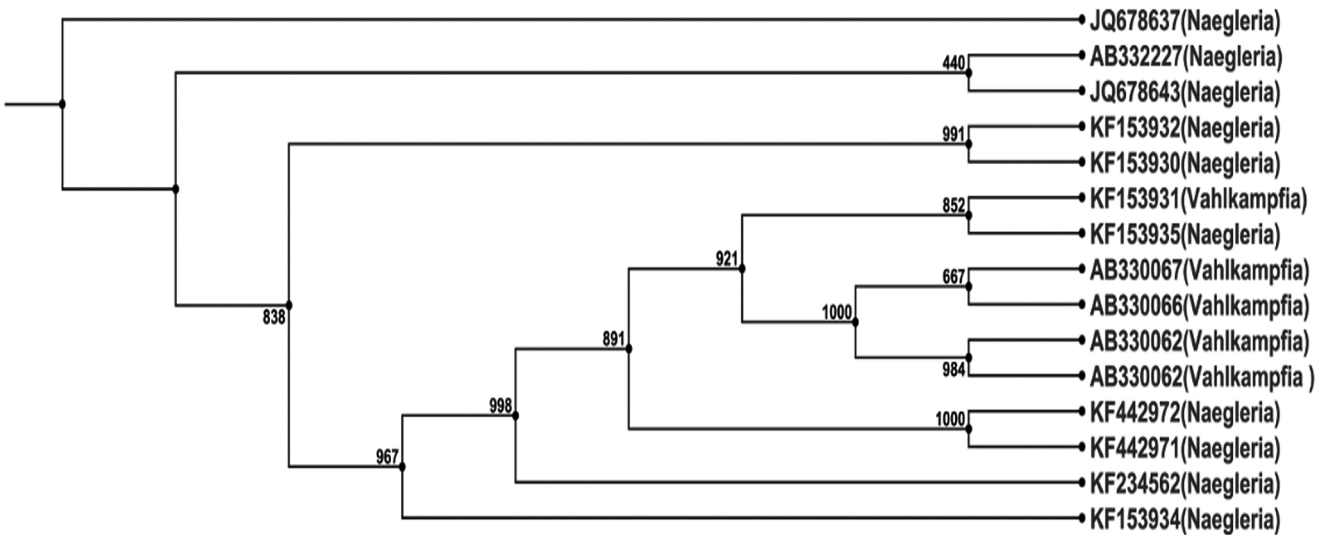


Fig. 3. To confirm the incidence of *Naegleria* populations in water and soil samples, DNA were extracted from amoebae retrieved from NNA plates with 2 weeks time and utilized for PCR examination as demonstrated in methods section. PCR products were obtained in all DNA samples verifying the existence of *Naegleria*. Lane 1: 250 bp DNA ladder; Lane 2: RAW STW4; Lane 3: RAW LW1; Lane 4: RAW PW8; Lane 5: RAW DS1; Lane 6: RAW STW7; Lane 7: RAW TW3; Lane 8: RAW DS2; Lane 9: RAW DS3; Lane 10: RAW DS4; Lane 11: RAW DS5; Lane 12: RAW DS6; Lane 13: RAW DS7; Lane 14: RAW DS8; Lane 15: RAW DS9; Lane 16: RAW DS10; Lane 17: +ve control; Lane 18: -ve control.

(A)



(B)

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AB330066(Vahlkampfia)  --CGACGATG AGGGATCAGG TGCTTAGCTT CCGTTGAGAG CGCCTTT-GG
AB330067(Vahlkampfia)  --CGACGACG AGGGACCAGG TGCTTAGCTT CCGTCGAGAG CGCCTCA-GG
AB330062(Vahlkampfia ) --CGACGATG AGGGATCAGG TGCTTAGCTT CCGTCGAGAG CGCCCCTCGG
AB330062(Vahlkampfia)  --CGACGATG AGGGATCAGG TGCTTAGCTT CCGTCGAGAG CGCCCCTCGG
KF153935(Naegleria)    --CGACGATG AGGGATCAGG TGCTTAGCTT CCGTCGAGAG CGCCCCTCGG
KF153931(Vahlkampfia)  --CGACGATG AGGGATCAGG TGCTTAGCTT CCGTCGAGAG CGCCCCTCGG
KF442971(Naegleria)    AGTGCTTGCA CAGGCACATC TATTTGAGTG TTGGTAA--- -----ATTAT
KF442972(Naegleria)    AACAGTAATG CTAGCACACT TGTTTGAGTG TCGTTAA--- -----TTA--
JQ678643(Naegleria)    --TGGTAAAA GGTGTTTGAA TCGTTAGGTT CTTACGCCCT AGCCGGTGTA
JQ678637(Naegleria)    --TGGTAAAA GGTGTTTGAA TCGTTAGGTT CTTACGCCCT AGCCGGTGTA
AB332227(Naegleria)    --TGGTAAAA GGTGTTTGAA TCGTTAGGTT CTTACGCCCT AGCCGGTGTA
KF153930(Naegleria)    --TGGTAAAA GGTGTTTGAA TCGTTAGGTT CTTACGCCCT AGCCGGTGTA
KF153932(Naegleria)    --TGGTAAAA GGTGTTTGAA TCGTTAGGTT CTTACGCCCT AGCCGGTGTA
KF153934(Naegleria)    --TGGTAAAA GGTGTTTGAA TCGTTAGGTT CTTACGCCCT AGCCGGTGTA
KF234562(Naegleria)    AATTGTGATG AGAATTGAAG TATTTAAC-- -------AAA TGCTTCTGAT
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Fig. 4. (A) Primary sequence alignment was obtained with reference sequences already available in Gen Bank. 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. **(B).** 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).

be interesting to further evaluate the composition of decomposed soil and other microbial flora which supports *Naegleria* growth. One of the major reasons behind this quick report is that all the reported samples were collected from 4km radius of the area. This enforce the point that this area is affluent with FLA and further detailed investigation is necessary to record all possible species of *Naegleria* along with other FLA like *Acanthamoeba*, *Balamuthia* and *Hartmannella* which could be a potential risk to public in future. In this regard we have lately identified and molecular characterized *Acanthamoeba* species from various water sources across Pakistan (Tanveer et al. 2015; Tanveer et al. 2013).

According to the reports worldwide single species of FLA, *Naegleria fowleri* is responsible for PAM but none of the isolates could be identified as *Naegleria*

fowleri during this study, which is quite surprising. On the other hand, the widespread existence of thermotolerant *Naegleria* in diverse water supplies, particularly *Naegleria lovanensis* (which is an indicator species for *Naegleria fowleri* presence) suggests this pathogenic amoeba might cause hazard to public health in the study area (Marciano-Cabral and Cabral 2007). In this context, our research group has already successfully recovered two thermotolerant species i.e. *Naegleria australiensis* (pathogenic) and *Naegleria lovaniensis* (non-pathogenic) from Pakistan (Tanveer et al., 2017) suggesting the possibility of *Naegleria fowleri* in various environmental sources of Pakistan.

FLA has been isolated from normal and synthetic marine habitats and even from tap water (Schuster and Visvesvara 2004b; Tanveer et al. 2017). It is generally acquired while swimming and diving in freshwater lakes and ponds (Martinez and Visvesvara 1997). Isolation of *Naegleria* (both soil and water sources) from this area can be potentially a future risk for the inhabitants and the tourists in particular as the latter are mainly involve the water sports in summer holidays. To the best of our knowledge, this is the first study, demonstrating the isolation and identification of *Naegleria* from water and soil samples of Rawlakot, Azad Jammu and Kashmir, Pakistan. It has been notified from previous published reports that seasonal variation does affect the prevalence of the amoebae in the environments (John and Howard 1995). Furthermore, FLA has been retrieved from a wide range of water temperatures from 0 to 45°C and at neutral pH (John and Howard 1995; Tyndall et al. 1989; Brown et al. 1983). In this context, our next step would be to collect further water and soil samples across AJK, Pakistan during five seasons (autumn, monsoon, spring, summer and winter) of the year along with pH and temperature records of both media (at time of sample collection) to know the exact micro flora (particularly FLA) in various environmental sources of the country throughout the year.

CONCLUSION

Overall, this study revealed soil and water both medium are affluent of *Naegleria* species which perhaps a possible hazard for human health and could also act as a proliferation means of other pathogenic bacteria in diverse natural resources of Pakistan. *Naegleria* has been reported from diverse settings worldwide, but not a single case of PAM infection has been reported from the subject area to date. This may be due to lack of expertise, lack of interest of the researcher in FLA and lack of awareness among local community including the clinician.

Our future study will be more focused on the further characterization of the identified species and analysis of household drinking water of the area, as *Naegleria fowleri* has been reported to cause infections in human through drinking water (tap water) in Karachi. Future investigations are required to establish the pathogenic potential of the identified *Naegleria* species and their function in the ecosystem. There is an urgent need to raise awareness among clinicians and general public about the risk of future amoebic infections itself and other pathogenic bacterial infections after being hosted by amoeba.

Overall, amoebic research is very limited worldwide and almost none in the Third World Countries (especially in Pakistan). In this context, the author draws attention to the relevant funding agencies, research and education institutions to promote amoebic research worldwide which eventually enhance our knowledge about upcoming various FLA threats. The author also encouraged to launch an awareness campaign about FLA and PAM through electronic and social media to local and scientific community across the country.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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