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PCR-based identification of thermotolerant free-living amoebae in Italian hot springs

Federica Berrilli [a,*], David Di Cave [a], Andrea Novelletto [b], Margherita Montalbano Di Filippo [a]

[a] *Department of Clinical Sciences and Translational Medicine, University of Rome Tor Vergata, Via Montpellier 1, 00133 Rome, Italy*
[b] *Department of Biology, University of Rome Tor Vergata, Via della Ricerca Scientifica 1, 00133 Rome, Italy*

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Abstract

Several thermal areas, also used for leisure purposes, may represent suitable habitats for free-living amoebae (FLAs), but few studies have been carried out in search for these organisms. The aim of this study was to assess the presence and distribution of FLAs by culture detection and molecular identification, over a one year-round sampling of two sites in Central Italy. Two geothermal springs (Site A and Site B) were investigated for a total of 36 water samples. Four sets of primers were used to amplify FLA DNA from all cultures positive for amoebic growth at both 37 °C and 45 °C. Overall, 33 (91.6%) water samples produced PCR amplification. Eleven taxa were identified. The array of identified species varied over the sampling period, and differed between the two hot springs, Site A harbouring 11 taxa compared to 5 of site B. However, both sites were characterized by the most common species *Vermamoeba vermiformis* and *Naegleria australiensis*. *Acanthamoeba* genotypes T4 and T15 were found at low frequency. Differences in the composition between the two sites could reflect environmental changes in biotic and chemical/physical parameters. From a public health perspective, the detection of potentially pathogenic amoebae could unveil a potential risk for humans.

Keywords: Free-living amoebae; Potentially pathogenic species; Molecular identification; Community composition; Geothermal springs; Italy

Introduction

Thermal environments represent suitable places to be inhabited by specific microbial communities comprising Archaea, Bacteria and Eukarya, due to the interplay of several abiotic and biotic factors (e.g., temperature, pH, nutrient levels, and biofilm texture) at levels permissive for the survival of these organisms. Among protists, the so-called free-living amoebae (FLAs) represent an historical assemblage, lasting from the times when Heterolobosea were a part of Gymnamoebia (Page 1988). Taxonomically, amoebae and amoeboid organisms can be found in a majority of eukaryotic "Supergroups" comprising a large number of taxa, studied to a variable extent. The amoebae presented in this paper fall within the Supergroups Amoebozoa and Excavata (Adl et al. 2019).

Despite their taxonomic and phyletic heterogeneity, free-living amoebae as a whole have gained growing interest in

*Corresponding author at: Department of Clinical Sciences and Translational Medicine, Faculty of Medicine, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy.
E-mail address: berrilli@uniroma2.it (F. Berrilli).

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the context of communities of thermal waters, due to their ability to survive and replicate without a host, also by forming resistant cysts. Indeed, FLAs appear more frequently in thermally enriched water collections, thermally polluted discharge waters from industrial plants and lakes or swimming pools, while non-thermally enriched water reserves seem to contain less potentially pathogenic species (Martinez, 1985; Lares-Jiménez et al., 2018; Latifi et al., 2020). Usually, FLAs do not threaten humans; however, some genera are known to be potential parasites of a wide range of vertebrates, including humans, able to cause severe and potentially lethal infections of the eye, the skin and the central nervous system. These organisms, named "amphizoic amoebae", have a dual biological characteristic, (i) an exozoic, non-parasitic phase, capable of transforming into (ii) an endozoic parasitic stage (Page 1974). Among them, *Acanthamoeba* spp. is the most frequent genus causing amoebic keratitis (AK) and other diseases in humans (Page, 1988; Visvesvara et al., 2007). *Vermamoeba vermiformis* has been isolated from the cerebrospinal fluid of a patient with meningoencephalitis (Centeno et al. 1996), also representing a suitable host of pathogenic bacteria (Santic et al. 2011). This species, as well as *Vahlkampfia* spp., may also cause amoebic keratitis (AK) (Aitken et al., 1996; Alexandrakis et al., 1998; Inoue et al., 1998; Lorenzo-Morales et al., 2007; Pinna et al., 2017). *Balamuthia mandrillaris* and *Naegleria fowleri* cause central nervous system infections, i.e. granulomatous amoebic encephalitis (GAE) and primary amoebic meningoencephalitis (PAM), respectively (Visvesvara et al., 2007; De Jonckheere, 2014).

Infections caused by these ubiquitous organisms remain rare, but are being reported at a growing pace in the last decades worldwide, due to a combination of different factors (e.g. increased freshwater and thermal recreational activities during heat waves, and growing numbers of soft contact lens wearers at risk of amoebic keratitis) (Acharya et al., 2007; Carnt and Stapleton, 2016). Studies on the epidemiology of FLAs in thermal waters have been carried out all over the world (Fabres et al., 2016; Gianinazzi et al., 2010; Lekkla et al., 2005; Rivera et al., 1989). In Italy, few data on FLA distribution are available, despite the widespread occurrence of thermal springs, often used for leisure purposes.

The aim of this study was to determine the composition of thermotolerant free-living amoebae communities, using culture-confirmed detection, over a year-round sampling in two hot springs used as recreational areas in Central Italy. As the range of morphological characters suitable for a specific diagnosis of free-living amoebae is rather limited and only some genera can be recognized in the light microscope (Dyková and Lom 2004), a molecular approach using different genomic regions as molecular markers, was performed.

Material and Methods

Study area

The present investigation was conducted in northern Latium (Central Italy), a region featuring a constellation of thermal areas with numerous hot springs and pools. In particular, the Viterbo area features volcanic ground waters belonging to the same recharge area of the Cimini Mountains-Lake Vico and characterized by a wide range of values for thermal (30–65 °C) and chemical (pH from 5.8 to 7.2) parameters (Piscopo et al., 2006; Valeriani et al., 2018).

Two geothermal springs of the sulphureous-sulphate-bi carbonate-alkaline-earth water type, used for wellness and recreational purposes, were investigated. Site A consists of four natural pools, fed by a hot water source with an average outlet temperature of 58 °C, an electrical conductivity of 2900 µS/cm, and CO_2 and H_2S contents of 350 mg/l and 15 mg/l, respectively. Site B is made up of a natural thermal pool connected through concrete and PVC piping to a terminal concrete swimming pool. The hot water source consists of a natural thermal bore located approximately 200 m upstream from the pools. Water springs out at a temperature from 57 °C to 60 °C, with an electrical conductivity of 3000 µS/cm, and CO_2 and H_2S contents of 200 mg/l and 9.6 mg/l, respectively.

Sampling and culture of FLAs

A single water sample was harvested quarterly from April 2015 to July 2016 from each pool of both sites (4 pools for Site A and 2 pools for the Site B), for a total of 36 water samples collected during the four seasons (Table 1).

Water temperature and pH were measured during each sampling using a thermometer and a digital pH meter, respectively (refer to Table 1). At each site location, two liters of water were collected in sterile flasks, transported to the laboratory at ambient temperature and processed up to 48 hrs. As this study was focused on thermotolerant cyst-forming FLAs, we considered this time span acceptable to avoid drastic changes in the FLA content of our water samples.

After centrifugation at 3000 rpm for 20 min, the pellet was re-suspended in 200 µl of supernatant and 100 µl aliquots were plated in double onto a Non-Nutrient Agar (NNA) with a thin layer of *Escherichia coli* laboratory strain (K-12). The two plates were incubated at 37 °C and 45 °C, respectively, and observed daily under an inverted light microscope at 200× and 400× magnifications for thermotolerant amoebic growth.

Table 1. Temperature and pH of 36 thermal water samples and FLA species growth in culture at 37 °C and 45 °C, by sampling session.

Season	Study Area		Temperature (°C)	pH	FLA isolates (ID*)	
	Site	Sampling points			Growth at 37 °C	Growth at 45 °C
Spring_15	A	P1	39.1	5.5	-	-
		P2	37	6	PC:04/15 P237_Ve	-
		P3	43.3	6.7	-	-
		P4	39.4	6.5	PC:04/15 P437_Ve PC:04/15 P437_Na	PC:04/15 P445_Fu
	B	PB	38.5	6.4	TB:04/15 PB37_Ve TB:04/15 PB37_Na	-
		PC	38	6.4	-	-
Summer_15	A	P1	34.8	8	PC:07/15 P137_Na	-
		P2	39.1	8	PC:07/15 P237_Un PC:07/15 P237_Na	-
		P3	48.4	8	-	-
		P4	40.8	8	PC:07/15 P437_A15 PC:07/15 P437_Ve PC:07/15 P437_Na	-
	B	PB	38.7	8	-	-
		PC	31	7	-	-
Autumn_15	A	P1	36.7	8	PC:10/15 P137_St PC:10/15 P137_Na	-
		P2	43.2	8	PC:10/15 P237_Na	-
		P3	37.8	8	PC:10/15 P337_Na	-
		P4	38	7	PC:10/15 P437_Ve PC:10/15 P437_Na	-
	B	PB	37.6	7	TB:10/15 PB37_Na	-
		PC	27.6	7	-	-
Winter_16	A	P1	29.9	7	PC:01/16 P137_Ec PC:01/16 P137_Na	PC:01/16 P145_Nl
		P2	36.6	7	-	PC:01/16 P245_Nl
		P3	39.4	7	PC:01/16 P337_Un PC:01/16 P337_Na	-
		P4	43	8	PC:01/16 P437_Un PC:01/16 P437_Na	PC:01/16 P445_N
	B	PB	22.5	7	TB:01/16 PB37_Un TB:01/16 PB37_Ni	-
		PC	11.3	7	-	-
Spring_16	A	P1	34.6	7	PC:04/16 P137_Na	-
		P2	39	8	PC:04/16 P237_Un PC:04/16 P237_Na	PC:04/16 P245_Nl
		P3	41.5	7	PC:04/16 P337_Nl	-
		P4	38.9	7	PC:04/16 P437_Va PC:04/16 P437_Na PC:04/16 P437_Ni	-
	B	PB	37.2	7	TB:04/16 PB37_A4 TB:04/16 PB37_Ve TB:04/16 PB37_Na	TB:04/16 PB45_Ve
		PC	19.7	7	TB:04/16 PC37_Ve TB:04/16 PC37_Na	-

(*continued on next page*)

Table 1 (*continued*)

Season	Study Area		Temperature (°C)	pH	FLA isolates (ID*)	
	Site	Sampling points			Growth at 37 °C	Growth at 45 °C
Summer_16	A	P1	36	7	PC:07/16 P137_A15	PC:07/16 P145_Ve
		P2	41.8	8	PC:07/16 P237_Ve PC:07/16 P237_Nl	PC:07/16 P245_Ve
		P3	45.8	8	-	-
		P4	38.4	7	PC:07/16 P437_A15 PC:07/16 P437_Ve	PC:07/16 P445_A15 PC:07/16 P445_Ve PC:07/16 P445_Va
	B	PB	41.2	7.5	-	-
		PC	30.4	7	-	-
		Total = 36			**Prevalence 66% (24/36)**	**Prevalence 25% (9/36)**

*Each isolate is labeled (ID) according to: the site (Site A = PC; Site B = TB), month and year of isolation (mm/YY), sampling point, culture growth temperature and identification (A4 = *Acanthamoeba* genotype T4; A15 = *Acanthamoeba* genotype T15; E = *Echinamoeba* sp.; Fu = *Fumarolamoeba* sp.; Un = "Uncultured" amoeba; Ve = *Vermamoeba vermiformis*; St = *Stenamoeba* sp.; Na = *N. australiensis*; Ni = *N. italica*; Nl = *N. lovaniensis*; N = unassigned *Naegleria* species; Va = *Vahlkampfia* sp.).

DNA extraction, PCR and sequence analysis

The growing amoebae were scraped from positive plates, transferred in Eppendorf tubes and washed twice in sterile phosphate buffered saline (PBS), pH 7.4. Genomic DNA was isolated using the QIAamp DNA Micro Kit (Qiagen, Milan, Italy), following the manufacturer's instructions.

All positive samples were amplified by using four sets of primers, which can recognize different FLA taxa, according to previous descriptions: (i) *Acanthamoeba* genus-specific JDP1 (5′ - GGC CCA GAT CGT TTA CCG TGA A – 3′) and JDP2 (5′ - TCT CAC AAG CTG CTA GGG AGT CA – 3′) designed to amplify a 18S rDNA region defined as ASA.S1 that includes the hypervariable diagnostic fragment 3 (DF3) (Schroeder et al. 2001); (ii) *Naegleria* genus-specific F (5′- GAA CCT GCG TAG GGA TCA TTT –3′) and R (5′- TTT CTT TTC CTC CCC TTA TTA –3′) designed from the rDNA ITS regions as described previously (Pélandakis et al. 2000); (iii) *Naegleria fowleri* species-specific (5′- TTC CGA ACC CAC TCA ATA AA – 3′ and 5′ - TTC CGA ACC CTT AAA ACC TC – 3′) targeting a single-copy DNA region (Régoudis and Pélandakis 2016); (iv) the 18S rDNA primers P-FLA-F (5′ - CGC GGT AAT TCC AGC TCC AAT AGC – 3′) and P-FLA-R (5′ - CAG GTT AAG GTC TCG TTC GTT AAC – 3′), which amplifies other FLA species (for more details refer to Tsvetkova et al. 2004).

PCRs were carried out in a 25 µl volume, containing 12.5 µl PCR master mix 2X (Promega, Milan, Italy), 5 µl template DNA, and 0.6 mM of each primer, in a TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany). All PCRs included negative and positive controls, particularly samples collected at the Tor Vergata University and previously characterized as *Acanthamoeba* spp., *Naegleria* spp., and other FLA species. Amplification products were visualized by electrophoresis on 1.5% agarose gels, stained by SYBR Safe DNA gel stain (Invitrogen, Monza MB, Italy) and amplicons were purified using the mi-PCR Purification Kit (Metabion GmbH, Steinkirchen, Germany) according to the manufacturer's instructions, and shipped to Bio-Fab Research (Rome, Italy) for sequencing.

DNA chromatograms were examined using FinchTV 1.4 (Geospiza, Inc, Seattle, WA, USA). After adjoining the forward and reverse reads, the assembled sequences were used to search GenBank using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.gov/BLAST/) to identify sequences identities and/or similarities (>95% of identities).

Results

Amoebic growth was observed in samples collected all year round, but mainly in spring 2016, when all sampling points (4 for the Site A and 2 for the Site B) resulted positive. Conversely, in summer 2015 and 2016, no positivity was detected in Site B. In detail, FLA growth was observed in 79% (19/24) of plates incubated at 37 °C and 33.3% (8/24) at 45 °C from Site A and in 41.66% (5/12) of plates at 37 °C and 8.3% (1/12) at 45 °C from Site B (Table 1).

Overall, DNA from 33 out of 36 water samples (91.6%) produced PCR amplification with three out of four sets of primers (generic FLA primers and *Acanthamoeba* and *Naegleria* genus-specific primers). In particular, 54 sequences were obtained from 24/36 (66%) cultures incubated at 37 °C and from 9/36 (25%) incubated at 45 °C. *N. fowleri* species-specific primers did not produce any amplicon band against all isolates.

Analysis of the 54 sequences revealed 25 isolates assigned to the Supergroup Amoebozoa, whereas the remaining 29 were attributed to the Supergroup Excavata. Table 2 displays the sequences (n = 14) originally characterized in this paper, with their putative taxonomic assignment. Table 3 lists the remaining sequences (n = 40) previously described (Corsaro et al., 2017; Montalbano Di Filippo et al., 2017, 2019). Among Amoebozoa we were able to recognize *Acanthamoeba* genotypes T4 and T15, *Vermamoeba vermiformis* and two isolates possibly attributable to the genera *Echinamoeba* (99.18% sequence identity) and *Stenamoeba* (96.13%), respectively (Tables 2 and 3). Taxonomic assignment was more difficult for five isolates showing 98.29 to 98.51% identity with an "uncultured eukaryote clone" (LN585909), putatively related to the genus *Echinamoeba* (Table 2).

Regarding the supergroup Excavata, we identified one isolate as *Fumarolamoeba* sp. (99.64% sequence identity), two as *Vahlkampfia* sp. (98.74–99.71% sequence identities), and several isolates assigned to different species of *Naegleria* (Tables 2 and 3).

More than one amoeba species was identified in cultures from some water samples, either at the same or different temperatures (for more details see Table 1). Four taxa (*Acanthamoeba* genotype T15, *V. vermiformis*, *Naegleria lovaniensis* and *Vahlkampfia* sp.) grew at both incubation temperatures. *Echinamoeba* sp., *Stenamoeba* sp. and the "uncultured" amoebae isolates grew exclusively at 37 °C.

V. vermiformis, the "uncultured" amoebae isolates, *Naegleria australiensis* and *Naegleria italica* were detected in both sites A and B, as well as representatives of the genus *Acanthamoeba*. However, *Acanthamoeba* genotype T15 was isolated twice in summer (2015 and 2016) only from Site A, while *Acanthamoeba* genotype T4 was detected once in spring 2016 in Site B. *N. lovaniensis* and *Vahlkampfia* sp. were found only in Site A.

Species number varied over the sampling period and differed between the two hot springs. Indeed site A turned out to harbour 11 taxa as compared to the 5 of site B. Both sites shared the presence of *V. vermiformis* and *N. australiensis*, i.e. the species most frequently recovered all year round throughout the study area.

Table 2. Sequences originally described in the present study.

Isolate ID	Primer set producing amplification	Sequence Identity with Reference Strain - Accession Number	Putative Taxonomic Assignment	Assigned Accession Number
TB:04/16PB37_A4	JDP1/JDP2 (Schroeder et al. 2001)	100% *Acanthamoeba* genotype T4 - MF399037	*Acanthamoeba* genotype T4	MT109098
PC:07/16 P445_A15	"	100% *Acanthamoeba* genotype T15 - GQ905495	*Acanthamoeba* genotype T15	NS*
PC:07/16 P437_A15	"	100% *Acanthamoeba* genotype T15 - GQ905495	"	MT109099
PC:07/16 P137_A15	"	100% *Acanthamoeba* genotype T15 - GQ905495	"	NS*
PC:10/15 P137_St	P-FLA-F/P-FLA-R (Tsvetkova et al. 2004)	96.13% *Stenamoeba stenopodia* - AY294144	*Stenamoeba* sp.	MT109100
PC:01/16 P137_Ec	"	99.18% *Echinamoeba exundans* - AF293895	*Echinamoeba* sp.	MT109101
TB:01/16 PB37_Un	"	98.43% "uncultured" eukaryote clone - LN585909	uncultured amoebae species	NS*
PC:04/16 P237_Un	"	98.51% "uncultured" eukaryote clone - LN585909	"	"
PC:01/16 P437_Un	"	98.38% "uncultured" eukaryote clone - LN585909	"	MT109102
PC:01/16 P337_Un	"	98.29% "uncultured" eukaryote clone - LN585909	"	NS*
PC:07/15 P237_Un	"	98.43% "uncultured" eukaryote clone - LN585909	"	"
PC:07/16 P445_Va	F/R (Pélandakis et al. 2000)	99.71% *Vahlkampfia* sp. AK-2007 clone RUI374- AB330066	*Vahlkampfia* sp.	MT109103
PC:04/16 P437_Va	"	98.74% *Vahlkampfia* sp. AK-2007 clone RUI374 - AB330066	"	NS*
PC:04/15 P445_Fu	P-FLA-F/P-FLA-R (Tsvetkova et al. 2004)	99.64% *Fumarolamoeba ceburocoi* - FR719837	*Fumarolamoeba* sp.	MT109104

* NS stands for NOT SUBMITTED.

Table 3. Isolates identified in Sites A and B described in previous publications.

Isolate ID	Taxonomic Assignment	Accession Number	Ref.
PC:07/15 P437_A15	*Acanthamoeba jacobsi* genotype T15	KY513796	Corsaro et al. (2017)
PC:04/15 P237_Ve	*Vermamoeba vermiformis*	NS*	Montalbano Di Filippo et al. (2019)
PC:04/15 P437_Ve	"	"	"
TB:04/15 PB37_Ve	"	"	"
PC:07/15 P437_Ve	"	"	"
PC:10/15 P437_Ve	"	MK110504	"
TB:04/16 PB37_Ve	"	NS*	"
TB:04/16 PC37_Ve	"	"	"
PC:07/16 P237_Ve	"	"	"
PC:07/16 P437_Ve	"	"	"
TB:04/16 PB45_Ve	"	MK110505	"
PC:07/16 P145_Ve	"	NS*	"
PC:07/16 P245_Ve	"	"	"
PC:07/16 P445_Ve	"	"	"
TB:04/15 PB37_Na	*Naegleria australiensis*	MF503260	Montalbano Di Filippo et al. (2017)
PC:07/15 P137_Na	"	NS*	"
PC:07/15 P237_Na	"	"	"
PC:07/15 P437_Na	"	"	"
PC:10/15 P137_Na	"	"	"
PC:10/15 P237_Na	"	"	"
PC:10/15 P337_Na	"	"	"
PC:10/15 P437_Na	"	"	"
TB:10/15 PB37_Na	"	"	"
PC:01/16 P137_Na	"	"	"
PC:07/15 P137_Na	"	"	"
PC:01/16 P337_Na	"	"	"
PC:01/16 P437_Na	"	"	"
PC:04/16 P137_Na	"	"	"
PC:04/16 P237_Na	"	"	"
PC:04/16 P437_Na	"	"	"
TB:04/16 PB37_Na	"	"	"
TB:04/16 PC37_Na	"	"	"
TB:01/16 PB37_Ni	*Naegleria italica*	MF503261	"
PC:04/16 P437_Ni	"	NS*	"
PC:01/16 P145_Nl	*Naegleria lovaniensis*	MF503262	"
PC:07/16 P237_Nl	"	NS*	"
PC:04/16 P337_Nl	"	"	"
PC:01/16 P245_Nl	"	"	"
PC:04/16 P245_Nl	"	"	"
PC:01/16 P445_N	*Naegleria* sp.	MF503263	"

* NS stands for NOT SUBMITTED.

Discussion

Due to the volcanic origin of a large proportion of the Italian territory, many geothermal springs can be counted, and thermal swimming pools, baths and spa for recreational use are widespread. As a result of the increase in the number of users, exposure and opportunity of contact with free living amoebae may be rising. However, few surveys on free living amoebae from thermal aquatic environments were previously carried out in Italy (Scaglia et al., 1983a, 1983b, 1987); the first two were performed in thermal pools and muds in North Italy, leading to the isolation of several FLA species: *N. australiensis* (23.3%), *N. italica* and *N. lovaniensis* (6%), *Acanthamoeba* spp. (5.2%), *Vahlkampfia* spp. (33.6%), and *Hartmannella* spp. (24.1%). In these studies, isolation was by culture and identification only by microscopy.

The present study represents the first comprehensive thermotolerant free-living amoebae investigation in hot springs accessed mainly for recreational purposes in Italy, carried out by culture combined with a PCR-based approach. Both the sampling and the culture steps of the experimental procedure here applied, may have reduced the real diversity of the surveyed water bodies because of

variable abundance (missing rare species), heterogeneity of the micro-environment (e.g. water column vs. sediments) and because our culture conditions may not be permissive for some, yet undetected, FLAs. On the other hand, our database searches returned identities with deposited sequences robustly assigned to known taxa in 97% of cases. The remaining cases (*i.e* the "uncultured" amoebae, *Echinamoeba* and *Stenamoeba* species – for more details see Table 2 in the results section) found matches with identities ranging from 96.13% to 99.18%, leaving little uncertainty to their assignment.

Overall, the arrays of species detected in the area under study were similar to those reported from these environments worldwide (Lekkla et al., 2005; Gianinazzi et al., 2010) and in other Italian sites (Scaglia et al., 1983a, 1983b, 1987), although spatial distribution of FLAs in the two sites here studied was variable. In particular, the genus *Naegleria* was the most frequently isolated: *N. australiensis*, widely distributed around the world (De Jonckheere, 2002, 2014) was the prevalent species (53%) detected in both sites, but only when culturing at 37 °C. *N. italica* and *N. lovaniensis* were identified, too, but with a lower prevalence. The pathogenic *N. fowleri* was not found. Although *N. australiensis* and *N. italica* have not yet been isolated from any human amoeba-related disease, different degrees of virulence were evidenced when tested in mice (Scaglia et al. 1983a). Furthermore, the presence of the thermophilic non-pathogenic *N. lovaniensis* could represent a good indicator of a favorable habitat also for *N. fowleri*, as the two species share similar environmental requirements (De Jonckheere, 2004; Schuster and Visvesvara, 2004). In Italy, the only case of fatal primary amebic meningoencephalitis (PAM) caused by *N. fowleri* was reported in a 9-year-old boy after swimming in a small water paddle associated with the Po River during a particularly hot summer (Cogo et al. 2004). However, all epidemiologic studies conducted in Italy on warm waters and thermal mud failed to isolate *N. fowleri*, indicating a very low risk of infection.

V. vermiformis was the second most frequently recovered species (33%), detected in both sites. This amoeba, of direct and indirect medical importance, has been described from many environments including geothermal springs at global scale (Bonilla-Lemus et al., 2014; Latifi et al., 2020; Solgi et al., 2012). In Italy, *V. vermiformis* isolates were previously identified from tap and ornamental fountain waters (Montalbano Di Filippo et al. 2015), confirming the wide distribution of this species in the country. Moreover, the presence of three genetic clusters of strains in the small thermal area here analysed, suggests environmental stability especially in the Site A, allowing to preserve genetic diversity of the *Vermamoeba* populations (Montalbano Di Filippo et al. 2019).

Conversely, it is remarkable that the genus *Acanthamoeba* was found at low frequency (19%). Recent studies focusing on the detection of this genus also in thermal waters, recorded prevalences of *Acanthamoeba* spp. ranging from 6.7% up to 50% (Fabres et al., 2016; Kao et al., 2012; Kiss et al., 2014). In our study, we isolated the potentially pathogenic *Acanthamoeba* genotype T4 once in spring 2016 in Site B, and *Acanthamoeba* genotype T15 twice in summer 2015 and 2016, in Site A. The genotype T15, described by Hewett et al. (2003) and associated with the species *Acanthamoeba jacobsi*, has been initially isolated only from environmental sources. In Italy, genotypes T4 and T15 related to AK have been previously described, representing the predominant *Acanthamoeba* genotypes involved in this serious infection (approximately 90% of cases) (Di Cave et al. 2009; 2014).

In addition, *Vahlkampfia* sp. was also here identified twice in this thermal area (Site A), growing at both temperatures, with a low prevalence (5%). *Vahlkampfia* is a free-living amoeba found in water and soil, and it belongs to the same family as *Naegleria*. It has been isolated in several investigations of human keratitis cases, but in none of them evidence that this genus actually caused the infection was considered conclusive. A fundamental problem arising from these reports is that in most cases these vahlkampfiid strains were not identified to the level of genus or species (as well as in this paper), perhaps because identification based on morphology is unreliable within the family Vahlkampfiidae (De Jonckheere and Brown, 2005). To date, *Vahlkampfia* spp. were previously reported in our country once in warm waters and thermal mud (Scaglia et al., 1982).

Among the non-pathogenic FLAs, we isolated once, only in Site A, *Echinamoeba* sp., *Stenamoeba* sp. and *Fumarolamoeba* sp. *Echinamoeba* and *Stenamoeba* have been occasionally reported from aquatic sources (especially freshwater) in other countries (Dyková et al., 2010; Gianinazzi et al., 2009, 2010; Page, 1969; Smirnov et al., 2007), while *Fumarolamoeba* was described for the first time in Mexico by De Jonckheere et al. (2011) and never reported in other epidemiological studies.

Differences in the numbers of taxa between the two springs observed during the year and in the different seasons could reflect environmental differences in the biotic (i.e. microbial structure) and chemical/physical parameters which characterize the ecosystem of the two sampling sites (Valeriani et al. 2018). In particular, in Site B, changes in the local management, such as the routine maintenance of the concrete pools, which periodically reduces the water level, may affect the amoebic biodiversity in spring.

From a public health perspective, the detection of the potentially pathogenic amoebae is of great interest. Considering that thermal springs are popular tourist attractions, and thousands of people swim in them, the wide distribution of potentially pathogenic FLAs in these water bodies underline the need of more frequent routine sampling necessary to better identify the potential risks for humans in our country.

CRediT authorship contribution statement

Federica Berrilli: Supervision, Data curation, Validation, Writing - original draft. **David Di Cave:** Funding acquisition, Resources, Writing - review & editing. **Andrea Novelletto:** Validation, Writing - review & editing. **Margherita Montalbano Di Filippo:** Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing - original draft.

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