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First report of free-living amoebae in watercourses in southern Brazil: molecular diagnosis and phylogenetic analysis of *Vermamoeba vermiformis*, *Naegleria gruberi*, and *Acanthamoeba* spp.

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ABSTRACT

Free-living amoebae (FLA) are protozoa dispersed in different environments and are responsible for different infections caused to humans and other animals. Microorganisms such as *Acanthamoeba* spp., *Vermamoeba* sp., and *Naegleria* sp. are associated with diseases that affect the central nervous system, in addition to skin infections and keratitis, as occurs in the genus *Acanthamoeba* and with *Vermamoeba vermiformis*. Due to the concerns of these FLA in anthropogenic aquatic environments, this work aimed to identify these microorganisms present in waters of Porto Alegre, Brazil. One litre sample was collected in two watercourses during the summer of 2022 and inoculated onto non-nutrient agar plates containing heat-inactivated *Escherichia coli*. Polymerase chain reaction results indicated the presence of FLA of the genera *Acanthamoeba, Vermamoeba*, and *Naegleria* in the study areas. Genetic sequencing indicated the presence of *V. vermiformis* and *Naegleria gruberi*. These aquatic and anthropogenic environments can serve as a means of spread and contamination by FLA, which gives valuable information on public health in the city.

Key words: *Acanthamoeba* spp., Dilúvio Stream, free-living amoebae, Guaíba Lake, *Naegleria gruberi*, *Vermamoeba vermiformis*

HIGHLIGHTS

- Identify microorganisms present in anthropogenic aquatic environments.
- First report of free-living amoebae in southern Brazil.
- Detection of *Acanthamoeba* spp., *Naegleria gruberi*, and *Vermamoeba vermiformis* in watercourses.
- These microorganisms are responsible for causing fatal diseases that affect the central nervous system.
- These environments can serve as a means of spread and contamination.

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GRAPHICAL ABSTRACT

1. INTRODUCTION

Free-living amoebae (FLA) are amphizoic protozoans. Some species of this group are known to cause severe diseases such as fatal encephalitis and cutaneous injuries (Visvesvara *et al.* 2007; Trabelsi *et al.* 2012). Because they are free-living organisms, some environments are more likely to be a source of contamination due to the easy exposure of people to the place, such as some water courses intended for recreational purposes, especially on hot days. They have been isolated from different natural and anthropogenic environments, from a wide range of aquatic and land habitats worldwide (Rodríguez-Zaragoza 1994; Visvesvara *et al.* 2009).

FLA have a cystic structure capable of resisting in unfavourable environmental conditions, which provides viability in chlorine-treated water, food shortages, dissection, extreme pH, and different temperatures. *Acanthamoeba*, a genus responsible for causing granulomatous amoebic encephalitis in immunosuppressed individuals and amoebic keratitis in healthy people, are easily found in swimming pools and lakes. In addition, some potential pathogens species of the other FLA, such as *Vermamoeba vermiformis* (famous for causing keratitis when associated with *Acanthamoeba*), *Naegleria fowleri* and *Naegleria gruberi* (which can cause a serious central nervous system infection called primary amoebic meningoencephalitis), are also isolated from various water matrices (Fowler & Carter 1965; Cerva 1969; Jager & Stamm 1972; Aitken *et al.* 1996; Scheid *et al.* 2019).

These organisms are considered the 'Trojan Horse' of other microorganisms (e.g., bacteria, fungi, and viruses) and can act as promoters or vehicles for the dissemination of pathogens called amoeba-resistant microorganisms, which can survive and multiply within the FLA. In this way, *Legionella pneumophila*, *Pseudomonas* spp., *Mycobacterium leprae*, *Candida auris*, and adenovirus can survive in unfavourable conditions and continue to present great pathogenic potential (Winiecka-Krusnell *et al.* 2009; Lovieno *et al.* 2010).

The study in question aimed to determine the occurrence of pathogenic and opportunistic FLA in two watercourses of great social and economic importance in Porto Alegre, southern Brazil. One of them, Guaíba Lake, is used as a water supply for some metropolitan cities. In addition, the other is a watercourse that runs through the capital city and flows into the lake. Due to the easy access to the population, knowledge about the quality of these courses is essential.

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2. METHODS

2.1. Geographical area and sample collection

In January 2022, a total of 17 samples were collected from the water samples of Guaíba Lake ($n = 9$) and Dilúvio Stream ($n = 8$) in Porto Alegre, the capital of the southernmost state of Brazil. Porto Alegre has a relatively cold and humid climate. Samples of 1 L of water were collected into 1,000 mL sterile flasks from all 17 points. All points were strategically preset. The first point collected in Guaíba Lake was in the south of Porto Alegre City, and Point 1 in Dilúvio Stream was the headwater located in the neighbouring city, Viamão-RS. Figures 1 and 2 show the sampling sites in each of them.

2.2. Samples process, culture, and cloning of FLA

All water samples were sedimented in a sedimentation cup. The sediment obtained after 24 h was centrifuged at 2,500 rpm for 10 min. Then, 200 μL of the sedimented material was transferred to a Petri dish containing 1.5% non-nutrient agar previously inoculated with heat-inactivated *Escherichia coli* (ATCC 8739). The dishes were inoculated at 30 °C for up to 40 days, while being examined daily and re-inoculated to eliminate any contamination by other microorganisms. Cloning, according to Diehl *et al.* (2021), was performed to obtain different isolates of FLA.

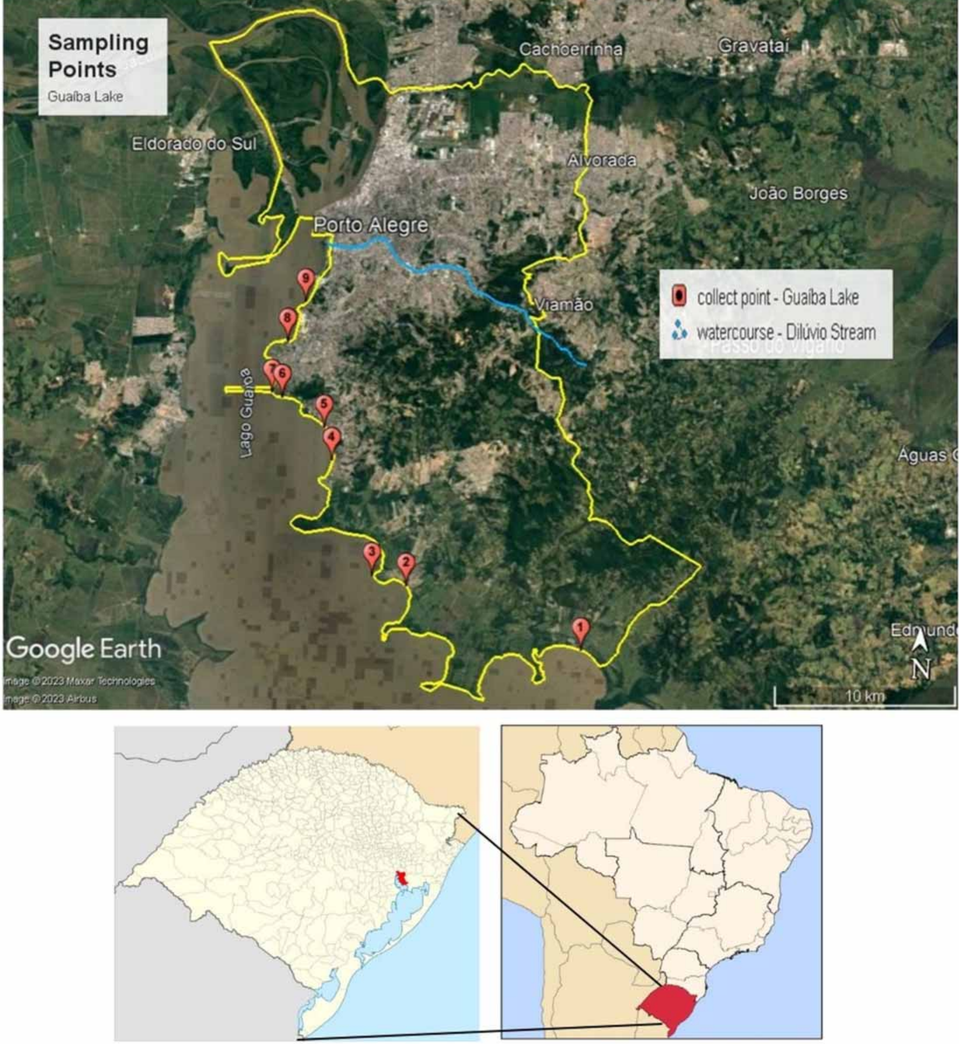

Figure 1 | Map containing the collection points in Guaíba Lake.

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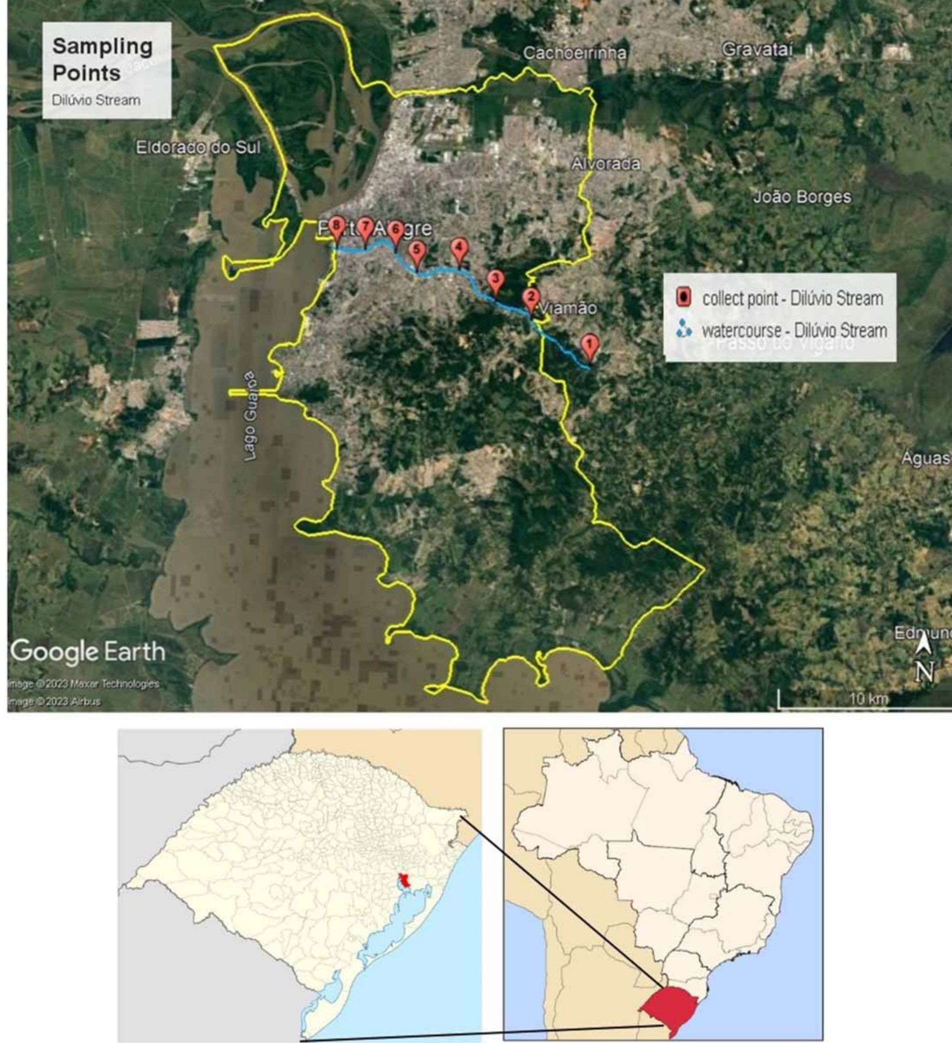

Figure 2 | Map containing the collection points in Dilúvio Stream.

2.3. DNA extraction

An extraction method capable of covering species of the Amoebidae family was developed. In this way, using a cell scraper and 3 mL of 1X phosphate-buffered saline (PBS), the amoebae were removed from the culture plates and subjected to 1,800rpm for 10 min. The pellet was resuspended in 500 μL of 1X PBS and homogenized. A total of 300 μL of the contents was transferred to a 2 mL Eppendorf, herewith 2.2 μL of proteinase K and 500 μL of 20% sodium dodecyl sulphate. After being homogenized by vortexing, the material was incubated in a water bath for 1.5 h at 60 °C. After the process, 800 μL of chloroform was added and vortexed. A total of 350 μL of protein precipitation (3M potassium acetate with 6.6 M glacial acetic acid) was added and shaken three times by hand. Then, the Eppendorf was centrifuged for 5 min at 11,000 rpm. The supernatant was transferred to a new 2 mL tube in which 1 mL of ice-cold absolute ethanol was added and homogenized by inversion for 2 min, to be later centrifuged at 11,000 rpm for 2 min. One millilitre of ice-cold 70% ethanol was added to the pellet and subjected to centrifugation again. The supernatant was discarded, and the pellet left to dry upside down for 10 min. After drying, 30 μL of Tris-EDTA buffer and 3 μL of RNAse were added to the microtube and incubated for 1 h at 37 °C. All extracted DNA was stored at −14 °C. The DNA was quantified using a nano spectrophotometer (Kasvi® K23-0002, version 01/13).

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2.4. Amplification and sequencing

To perform the amplification by polymerase chain reaction (PCR), three gene-specific oligonucleotides were used: JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′- TCTCACAAGCTGCTAGGGGATA-3′) for *Acanthamoeba* spp.; ITS1 (5′-GAACCTGCGTAGGGATCATTT-3′) and ITS2 (5′-TTTCTTTTCCTCCCCTTATTA-3′) for *Naegleria* spp.; and Hv1227F (5′-TTACGAGGTCAGGACACTGT-3′) and Hv1728R (5′-GACCATCCGGAGTTCTCG-3′) for *Vermamoeba* spp. The PCR reactions were performed with 10 pmol of each primer, 2.5 mM of deoxynucleoside triphosphate (DNTP), 50 mM of $MgCl_2$, 2.5 μL of 10X buffer, and 1 U of Taq polymerase (Invitrogen®) for a final volume of 25 μL. The PCR conditions were set to initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 60 °C for 40 s, and 72 °C for 1 min and 15 s (*Acanthamoeba* spp. according to Santos *et al.* 2022); 94 °C for 45 s, 58 °C for 40 s, and 72 °C for 30 s (*Vermamoeba* spp.); and 94 °C for 45 s, 55 °C for 40 s, and 72 °C for 1 min and 15 s (*Naegleria* spp. according to Henker *et al.* 2021). A final extension at 72 °C for 5 min was promoted in a SimpliAmp™ Thermal Cycler (Applied Biosystems). The negative control was performed with DNA-free, and the positive control was performed using a clinical isolate of *N. fowleri* (Henker *et al.* 2021) and *Acanthamoeba* spp. (Santos *et al.* 2022), and an environmental isolate of *V. vermiformis* (Soares *et al.* 2017). After PCR, the generated amplicons were submitted to electrophoresis and analysed on a 1.2% agarose gel.

The company ACTGene Molecular Analyzes performed sequencing using an ABI Prism 3500 Genetic Analyzer sequencer (Genetic Analyzer – Applied Biosystems®). The primers used were the same as in the PCR reaction, but, at this stage, they were tested separately for each reaction (only the forward primer or only the reverse primer) using 4.5 pM of each primer for a final volume of 6 μL containing 60 ng. The sequences forward and reverse identified were analysed and submitted to homology analysis in BLAST®, aligned by Clustal W 2.1, and deposited in the GenBank database.

2.5. Phylogenetic tree of the genera *Naegleria* and *Vermamoeba*

The phylogenetic analysis was based on the ITS for *Naegleria* spp. and 18 S rDNA for *Vermamoeba* spp. The evolutionary analyses were conducted in MEGA11 to build a phylogenetic tree based on neighbour-joining using the forward and reverse sequences. To determine the statistical reliability of each node, 500 bootstrap replicates were performed. This analysis involved 21 nucleotide sequences.

3. RESULTS

3.1. Clones of FLA

A total of 46 clones were obtained: 21 from Dilúvio Stream and 25 from Guaíba Lake. Some clones had sizes consistent with the genera studied.

3.2. PCR amplification and sequence analysis

Three clones from the Dilúvio Stream and one from Guaíba Lake were positive for *Acanthamoeba* spp. Eighteen clones were positive for *Vermamoeba* spp., 12 of which from Guaíba Lake. One isolated from Dilúvio Stream was positive for *Naegleria* spp. To represent each sampling point, the positive clones obtained in PCR were selected so that the distinct isolated genus (one or more) at each point could be sequenced. Figure 3 shows some examples of each found species.

Figure 3 | Positive clones by PCR with (a) the different polygonal cysts of *Acanthamoeba* sp. in 100X, (b) trophozoites of *Vermamoeba* sp. in 100X with its characteristic elongated morphology, and (c) a trophozoite of *Naegleria* sp. in 1,000X with a cylindrical body, emitting hyaline pseudopods during its movement.

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The sequences received from ACTGene were analysed in BLASTn to indicate the identity of the clones. Of these clones, only four reverted to a complete sequence that could be aligned together with BLASTn. Table 1 summarizes the results for all the samples with their respective registers in Genbank.

Our findings are demonstrated to be 93–98% identical with GenBank *N. gruberi* reference sequences, with a query coverage of 96–97% at BLASTn alignment. A total of 98–99% identical with *V. vermiformis* reference sequences with 95–97% of query coverage.

3.3. Phylogenetic analysis of *Naegleria* spp. and *Vermamoeba* spp.

Despite the molecular identification by PCR for the presence of *Acanthamoeba* in both study areas, the amplicons sent for sequencing were not reverted to complete sequences that could be aligned and identified by similarity in BLASTn, and therefore, it was not possible to carry out a phylogenetic tree of these.

Table 2 shows the reference sequences used for the construction of the phylogenetic tree with its relevant information.

The neighbor-joining was used as the method for building the phylogenetic tree. The bootstrap consensus tree was inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The icons indicate sequences referring to the same clone.

The sequences were used to create a neighbor-joining phylogenetic tree. Figure 4 shows the sequences aligned (forward and reverse) of AD4A_F and AD4A _ R, branching with other species of *N. gruberi* (A). The positive clones to *Vermamoeba* sp. with species as *Hartmannella vermiformis* (also known as *V. vermiformis*) (B).

Table 1 | Identity of clones

Sample site	Code	BLASTn/accession	Register on GenBank	Similarity (%)
Dilúvio Stream	AD4A_F	*Naegleria gruberi* MG699123.1	OP985783	98.92
	AD4A_R	*Naegleria gruberi* MG699123.1	OP994306	93.82
	AD4C_F	*Vermamoeba vermiformis* DQ407567.1	OP984080	99.78
	AD4C_R	*Vermamoeba vermiformis* MG969826.1	OP984113	99.56
	AD8C_F	*Vermamoeba vermiformis* MK418871.1	OP984114	98.68
	AD8C_R	*Vermamoeba vermiformis* MK418871.1	OP984115	98.90
Guaíba Lake	LG8B_F	*Vermamoeba vermiformis* MK418871.1	OP984125	99.78
	LG8B_R	*Vermamoeba vermiformis* MK418871.1	OP984126	99.78

The number accession indicates the sequence used to compare in BLASTn. The letters F and R indicate if the sequence analysed is forward or reverse, respectively.

Table 2 | Reference sequences used to build the phylogenetic tree

BLASTn/accession	Source	Strain/isolation source	Size (pb)
MG699123	*Naegleria gruberi*	EGB/river	14,007
AB298288	*N. gruberi*	NEG-M	14,128
AJ132022	*N. gruberi*	1518/1f	361
AJ132024	*N. gruberi*	NG273	322
JQ271648	*Naegleria* sp.	4542/liver	507
HE617186	*Hartmannella* sp.	V13/biofilm	694
GU001158	*H. vermiformis*	CR	4,410
MG969826	*V. vermiformis*	Hart15R/soil	475
DQ407567	*H. vermiformis*	CT1.3/cooling tower	502
KT185625	*V. vermiformis*	Isolate 1	7,278
MK418871	*V. vermiformis*	FREDDY/wound on the upper eyelid	1,795

Note: The sequences used for the construction of the phylogenetic tree were based on their similarity and/or similar isolation conditions to this work.

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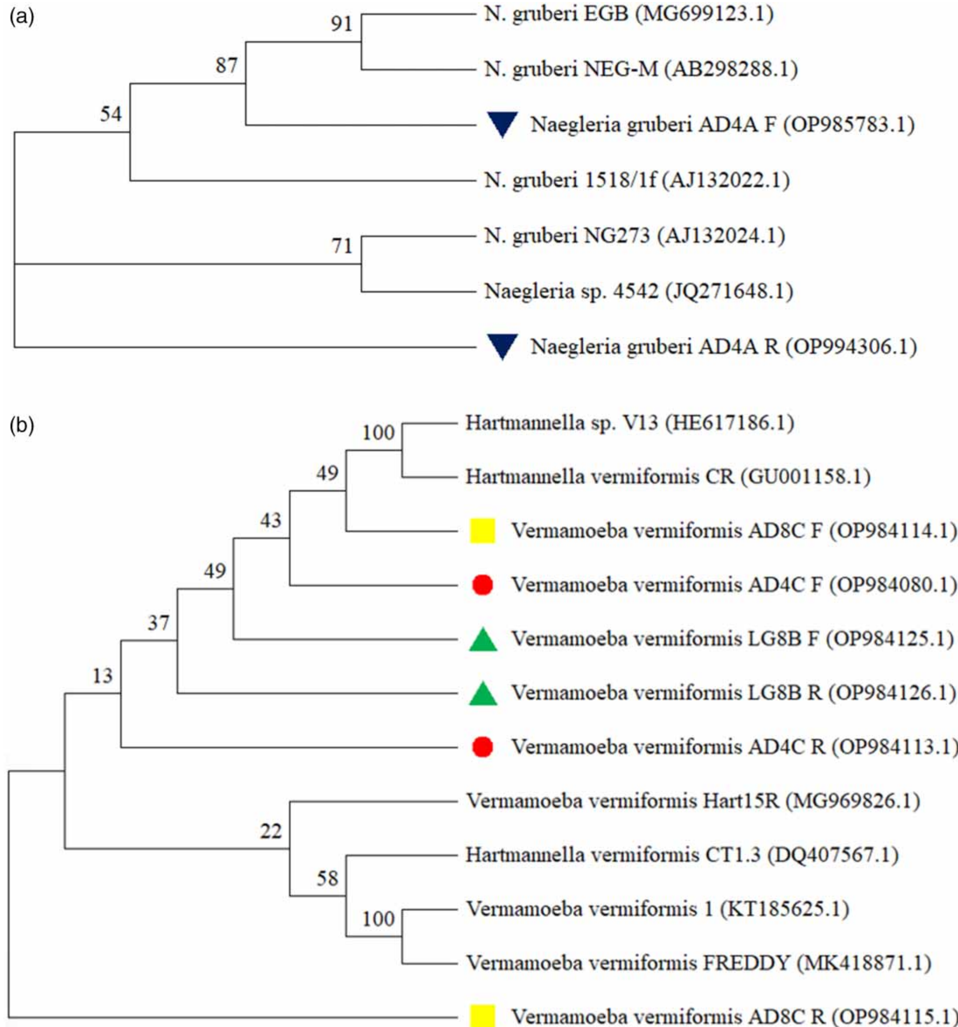

Figure 4 | The evolutionary relationship among *Naegleria* spp. and *Hartmannella* '*Vermamoeba*' spp. was based on internal transcribed sequences (ITS1 and ITS2 regions) and 5.8S sequence.

4. DISCUSSION

Of the 46 clones obtained, only 23 were positive for genera known to contain potentially pathogenic species. It is important to highlight that all clones have morphological features of FLA, such as amoeboid shape, presence of contractile vacuole, pseudopods, among others. But none showed dimensions compatible with *Sappinia* spp. and *Paravahlkampfia* spp. (in Supplementary Information – SI) and therefore were not tested for both genera. Although the isolation sites were in water, the clones were also tested for the *Balamuthia*, but all of them were negative.

The results show that the negative clones for the target genera in this study belong to non-potentially pathogenic FLA, though this does not exclude their ability to host other microorganisms with pathogenic characteristics, ensuring their protection in hostile environments and assisting in proliferation.

Almost all points on Guaíba Lake were positive for *Vermamoeba* spp., with the exception of the second and ninth sampling points. For *Acanthamoeba* spp., the fourth was the only positive site. Points 1, 7, and 8 of Dilúvio Stream were positive only for *Vermamoeba* spp., points 5 and 6 were positive for *Acanthamoeba* spp., and point 4 was positive for *Vermamoeba* spp. and *Naegleria* spp. Point 4, the only one where it was possible to isolate two genera, was the first completely urbanized site composed of residences and enterprises, in addition to being a point that precedes a hospital.

Javanmard *et al.* (2017), in their research that covered different water bodies, demonstrated *V. vermiformis* as the free-living amoeba most present there, ranking above other genera such as *Acanthamoeba* and *Naegleria*. Still, these authors related

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their results to cases of *V. vermiformis* keratitis reported in the same region where the study was carried out. Fani *et al.* (2022) observed the same result. Our results indicate that although there are no cases of infection by *V. vermiformis* reported yet, users of both watercourses studied are still subject to a possible infection.

The first report of *Naegleria philippineneis, Naegleria australiensis, Naegleria dobsoni*, and *N. gruberi* in South American environmental samples was described by Bellini *et al.* (2020). In this way, this is the first report of *V. vermiformis*, *N. gruberi*, and *Acanthamoeba* spp. in southern Brazil in an environmental sample. Although there are not many studies that point to *N. gruberi* as an agent of any disease, Cerva (1969) reported a case of an 11-year-old boy who died of acute amoebic meningoencephalitis caused by *N. gruberi*.

As a result, this study points to the presence of different potentially pathogenic microorganisms, one of them responsible for promoting or intensifying some other pre-existing diseases (*V. vermiformis*). These results demonstrate that these environments are ideal means for the promotion of diseases caused by FLA agents when in contact with a possible host.

5. CONCLUSION

It is important to point out that in Brazil, little environmental research has been carried out so far for a survey and mapping of pathogenic FLA in aquatic and anthropogenic environments. Furthermore, this is the first study on natural sources carried out in Rio Grande do Sul, southern Brazil. The study in question has epidemiological and environmental importance, since the genera found have species responsible for infecting both humans and other animals, in addition to carrying other pathogens. The Dilúvio Stream and Guaíba Lake, which are aquatic and anthropogenic environments often used as recreational facilities, can serve as a means for transmission and contamination by FLA, which gives valuable information on the public health in the city. Thus, we believe that some measures can be taken to mitigate future FLA infections. Using control measures, such as placing signage at the access points to these watercourses, is an effective strategy to promote awareness and safety. The signage can alert people to the risks associated with that specific area, providing important instructions and guidance due to the presence of free-living amoebae known to cause serious infections of the skin, cornea and central nervous system. These measures help prevent FLA infections and ensure that people adopt safe behaviors around watercourses. Additionally, it is crucial that the signs are clear, visible, and understandable to all users.

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AUTHOR CONTRIBUTION

All authors contributed to the study conception and design. The first draft of the manuscript was written by Brenda Teixeira Scardini Marinho and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Aitken, D., Hay, J., Kinnear, F. B., Kirkness, C. M., Lee, W. R. & Seal, D. V. 1996 Amebic keratitis in a wearer of disposable contact lenses due to a mixed *Vahlkampfia* and *Hartmannella* infection. *Ophthalmology* **103** (3), 485–494. https://doi.org/10.1016/S0161-6420(96)30667-2.

Bellini, N. K., Fonseca, A. L. M. d., Reyes-Batlle, M., Lorenzo-Morales, J., Rocha, O. & Thiemann, O. H. 2020 Isolation of *Naegleria* spp. from a Brazilian water source. *Pathogens* **9** (2), 1–13. https://doi.org/10.3390/pathogens9020090.

Cerva, L. 1969 Amoebic meningoencephalitis: axenic culture of *Naegleria*. *Science* **163** (3867), 576. https://doi.org/10.1126/science.163.3867.576.a.

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Diehl, M. L., Paes, J. & Rott, M. B. 2021 Single cell cloning of free-living amoebae with isolated square agar. *Parasitology Research* **120**, 3331–3333. https://doi.org/10.1007/s00436-021-07275-9.

Fani, M., Fuerst, P. A., Mosayebi, M., Javadi, A., Harandi, M. F., Saraei, M., Badri, M. & Hajialilo, E. 2022 Molecular identification and phylogenetic analysis of free-living amoeba in the water resources of Arak, Iran. *Journal of Water and Health* **20** (7), 1051–1063. https://doi.org/10.2166/wh.2022.030.

Fowler, M. & Carter, R. F. 1965 Acute pyogenic meningitis probably due to *Acanthamoeba* sp.: a preliminary report. *British Medical Journal* **2**, 740–743. https://doi.org/10.1136/bmj.2.5464.734-a.

Henker, L. C., Lorenzett, M. P., Santos, D. L. d., Virginio, V. G., Driemeier, D., Rott, M. B. & Pavarini, S. P. 2021 *Naegleria fowleri*-associated meningoencephalitis in a cow in Southern Brazil – first molecular detection of *N. fowleri* in Brazil. *Parasitology Research* **120**, 2873–2879. https://doi.org/10.1007/s00436-021-07209-5.

Jager, B. V. & Stamm, W. P. 1972 Brain abscesses caused by free-living amoeba probably of the genus *Hartmannella* in a patient with Hodgkin's disease. *The Lancet* **300** (7791), 1343–1345. https://doi.org/10.1016/S0140-6736(72)92781-X.

Javanmard, E., Niyyati, M., Lorenzo-Morales, J., Lasjerdi, Z., Behniafar, H. & Mirjalali, H. 2017 Molecular identification of waterborne free living amoebae (*Acanthamoeba, Naegleria* and *Vermamoeba*) isolated from municipal drinking water and environmental sources, Semnan province, north half of Iran. *Experimental Parasitology* **183**, 240–244. https://doi.org/10.1016/j.exppara.2017.09.016.

Lovieno, A., Ledee, D. R., Miller, D. & Alfonso, E. C. 2010 Detection of bacterial endosymbionts in clinical *Acanthamoeba* isolates. *Ophthalmology* **117** (3), 445–452. https://doi.org/10.1016/j.ophtha.2009.08.033.

Rodríguez-Zaragoza, S. 1994 Ecology of free-living amoebae. *Critical Reviews in Microbiology* **20** (3), 225–241. https://doi.org/10.3109/10408419409114556.

Santos, D. L. d., Virginio, V. G., Berté, F. K., Lorenzatto, K. R., Marinho, D. R., Kwitko, S., Locatelli, C. L., Freitas, E. C. & Rott, M. B. 2022 Clinical and molecular diagnosis of *Acanthamoeba* keratitis in contact lens wearers in southern Brazil reveals the presence of an endosymbiont. *Parasitology Research* **121**, 1447–1454. https://doi.org/10.1007/s00436-022-07474-y.

Scheid, P. L., Lâm, T., Sinsch, U. & Balczun, C. 2019 *Vermamoeba vermiformis* as etiological agent of a painful ulcer close to the eye. *Parasitology Research* **118** (6), 1999–2004. https://doi.org/10.1007/s00436-019-06312-y.

Soares, S. S., Souza, T. K., Berté, F. K., Cantarelli, V. V. & Rott, M. B. 2017 Occurrence of infected free-living amoebae in cooling towers of Southern Brazil. *Current Microbiology* **74**, 1461–1468. https://doi.org/10.1007/s00284-017-1341-8.

Trabelsi, H., Dendana, F., Sellami, A., Sellami, H., Cheikhrouhou, F., Neji, S., Makni, F. & Ayadi, A. 2012 Pathogenic free-living amoebae: epidemiology and clinical review. *Pathologie Biologie* **60** (6), 399–405. https://doi.org/10.1016/j.patbio.2012.03.002.

Visvesvara, G. S., Moura, H. & Schuster, F. L. 2007 Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunology & Medical Microbiology* **50** (1), 1–26. https://doi.org/10.1111/j.1574-695X.2007.00232.x.

Visvesvara, G. S., Sriram, R., Qvarnstrom, Y., Bandyopadhyay, K., Silva, A. J. d., Pieniazek, N. J. & Cabral, G. A. 2009 *Paravahlkampfia francianae* n. sp. masquerading as an agent of primary amoebic meningoencephalits. *The Journal of Eukaryotic Microbiology* **56** (4), 357–366. https://doi.org/10.1111/j.1550-7408.2009.00410.x.

Winiecka-Krusnell, J., Dellacasa-Lindberg, I., Dubey, J. P. & Barragan, A. 2009 *Toxoplama gondii*: uptake and survival of oocysts in free-living amoebae. *Experimental Parasitology* **121** (2), 124–131. https://doi.org/10.1016/j.exppara.2008.09.022.

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