Peer-reviewed version available at *Pathogens* **2020**, *9*, 90; doi:10.3390/pathogens9020090

Article

Isolation of *Naegleria spp.* from a Brazilian Water Source

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Abstract: The genus *Naegleria*, of free-living amoeba (FLA) group, has been investigated mainly due to its human health impact resulting in deadly infections and their worldwide distribution on freshwater systems. *Naegleria fowleri*, colloquially known as the "brain-eating amoeba", is the most studied *Naegleria* species because it causes Primary Amoebic Meningoencephalitis (PAM) of high lethality. The assessment of FLA biodiversity is fundamental to evaluate the presence of pathogenic species and the possibility of human contamination. However, the knowledge of FLA distribution in Brazil is unknown, and to rectify this situation we present a research on identifying *Naegleria spp.* in the Monjolinho River, as a model study. The river is a public Brazilian freshwater source that crosses the city of São Carlos. Five distinct sampling sites were examined through limnological features, trophozoites culturing and PCR against internal transcribed spacers (ITS) regions and 5.8S rRNA sequence. The results identified *N. philippinensis, N. canariensisi, N. australiensis, N.gruberi, N. dobsoni* sequences, as well as a *Vahlkampfia* sequence. The methodology delineated here represents the first Brazilian *Naegleria spp.* study on a freshwater system. Our result stresses the urgency of a large scale evaluation of the presence of free-living amoebas in Brazil.

Keywords: *Naegleria spp.*; free-living amoeba; PCR; Monjolinho River; Brazil

1. Introduction

Naegleria is a genus that comprises single-celled, heterotrophic protists widely distributed in natural environments [1–3]. The 47 species identified until now exist as free-living amoeba (FLA) with a bacteria based feeding habit and binary fission division [4]. In unfavorable environmental conditions they transform from trophozoites to the cyst stage, as a resting form [5]. Besides, a third stage, commonly biflagellate, can be formed to seek a better surrounding [6]. The known exceptions are *N. indonesiensis* and *N. chilensis* for which a flagellate form has never been identified [4].

Naegleria is described as an amphizoic genus as four species are able to thrive not only as free living organisms but also as parasites: *N. fowleri, N australiensis, N. philippinensis* and *N. italica* [7–9]. Since *N. fowleri* withstands higher temperatures as found in geothermal sources and heated recreational aquatic systems, the species has been classified as thermophilic [3,10] with the ability to grow in temperatures ranging from 30 ºC to 46 ºC [11,12]. Other species, *N. australiensis*, *N. italica*, *N. lovaniensis* and *N. philippinensis*, have been recognized as thermo tolerant as well [4,13].

Naegleria is phylogenetically grouped with another amphizoic genera, *Vahlkampfia*, at Vahlkampfiidae family, clade Discoba, super group Excavata [5,14–16]. In adittion, *Acanthamoeba, Balamuthia, Sappinia* and *Vermammoeba* genus are pathogenic FLA groups whose main threats to humans are granulomatous amoebic encephalitis (GAE), amoebic keratitis (AK) and primary amoebic meningoencephalitis (PAM) [17–19]. GAE is caused by *Acanthamoeba spp.*, *Balamuthia mandrillaris and Sappinia pedata* with several cases in immunocompromised persons whereas AK is caused mainly by *Acanthamoeba spp.* and *Vermammoeba vermiforms* with prevalence in contact lens wearers [19–21].

PAM is a fatal infection caused by *Naegleria fowleri* whose amoeboflagellate can reach the central nervous system (CNS) through the olfactory neuroepithelial pathway [22]. Seven to fourteen days after the appearance of symptoms 95% of the cases reported to date lead to death [18,24–26]. This alarmingly low survival rate has been correlated with the difficulty in diagnosis and the low efficacy of the available therapy, commonly based on antifungal and antibacterial drugs [27,28]. Additionally *N australiensis, N. philippinensis* and *N. italica* are potentially pathogenic species [7–9]. Considering that the amoeba infects the host by direct contact and that most of PAM patients have a history of water contact (e.g.: bathing in freshwater) prior the outcome of the encephalitis, the investigation on its environmental distribution is of great public health importance to prevent new cases and also critical to deepen the knowledge on *Naegleria* diversity.

Regarding *Naegleria* distribution throughout the world, the South American countries have presented a lack of information in contrast with more numerous data from European, North American and Asian researches [24]. In Brazil, the first occurrence of *N. fowleri* was reported in an artificial lake from the city of Rio de Janeiro [29]. Later the presence of *Naegleria* genus was linked to dust and biofilm from hospitals in the cities of Presidente Prudente [30] and Porto Alegre [31]. In 2009 *N. fowleri* was correlated to dust in two campuses of a university in the city of Santos [32]. Regarding Brazilian PAM cases, a 1985 report diagnosed the infection through immunological analysis of the cerebral tissue of a deceased patient [29] and two reports identified *N. fowleri* infection in cattle, based on histological and immunohistochemical analysis [33,34]. These reports lack molecular techniques to confirm the findings as a complementary strategy to combine with the morphological and physiological characterization of the isolated organism. Currently the ribosomal RNA gene, particularly the region of the internal transcribed spacers (ITS1, ITS2) and the 5.8S ribosomal gene, has been used in the newest studies of *Naegleria*.

Accentuating the lack of reports, Brazil is one of the most promising countries to *Naegleria* dispersion since it harbors about twenty percent of global freshwater [35], the most prevalent habitat in which *Naegleria* has been found. Taking into account this scenario on the biodiversity of *Naegleria* species, pathogenic or non pathogenic, in Brazilian freshwater courses, we have undertaken this initiative by investigating a river at the city of São Carlos, São Paulo state, as a model system. The Monjolinho River belongs to a basin that covers São Carlos city and neighboring districts in the state of São Paulo, and it reaches a watershed dimension of 275 km^2. The area belonging to São Carlos city is under intense anthropogenic activity and is highly impacted with domestic and industrial sewage, besides agricultural runoff [36,37]. Therefore, the present research examined five distinct sampling sites in which *Naegleria* spp. were isolated and characterized by PCR amplification and DNA sequencing of the ITS1, 5.8S and ITS2 region of total DNA from each site. Combined with the

morphological investigation after culturing and the limnological characterization of the water we were able to identify the presence of five *Naegleria spp.*a in Brazilian freshwater course.

2. Results

2.1. Limnologic characterization

OD, conductivity, pH and temperature measurements acquired in situ along the Monjolinho River are summarized in Figure 1.

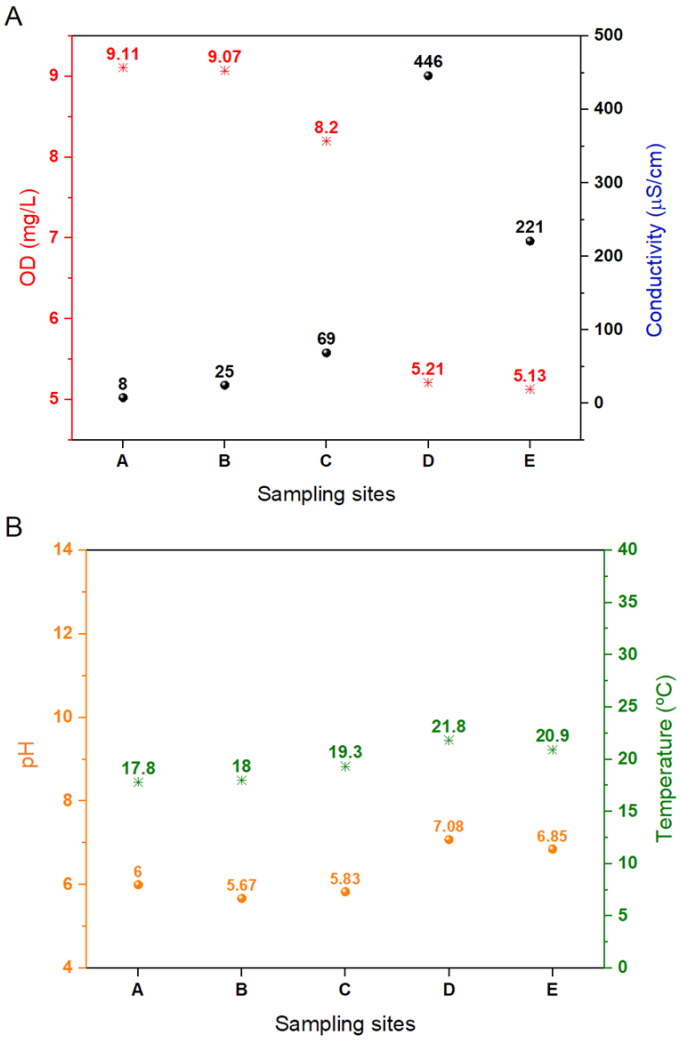

Figure 1: Limnological profile throughout the sampling sites in the Monjolinho River. (A) Correlation between OD and conductivity values. (B) Temperature and pH values plotted along the river.

Samples A to E along the river display a decrease in the dissolved oxygen concomitant with the increase in the conductivity values. The D position is striking by showing an abrupt accentuation on both data (Figure 1-A). These result correlates well with site D been located at a domestic sewage discharge area were organic compounds are dissolved in the water leading to high ionic concentration, consistent with a decrease in OD. On the contrary, the OD and conductivity values at sites A to C imply a lower effect of human activities, as well as site E, after the sewage treatment plant show a partial water quality recovery. Temperature and pH values are relatively constant from site A to E (Figure 1-B) and apparently are not influenced by the domestic sewage discharge. A small increase in temperature is observed, most likely due to daily variations in ambient temperature. As shown in Figure 1 (B), the recorded pH values ranged from 5.67 to 7.08 and temperatures from 17.8

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°C to 21.8 °C. Both limnological parameters were kept relatively constant along the five sampling sites.

2.2. Light microscopy

The daily microscopic examination of NNA plates allowed the identification of amoeba growth in 100% of the sampling sites covered in this study. According the amoeba cell morphologic profile defined by Page'S Key [38] the two life cycle stages, trophozoites and cyst, were observed (Figure 2).

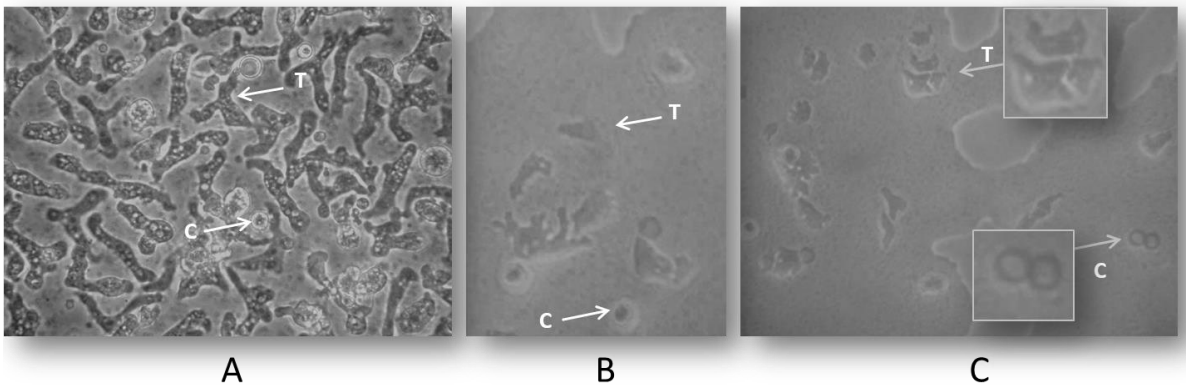
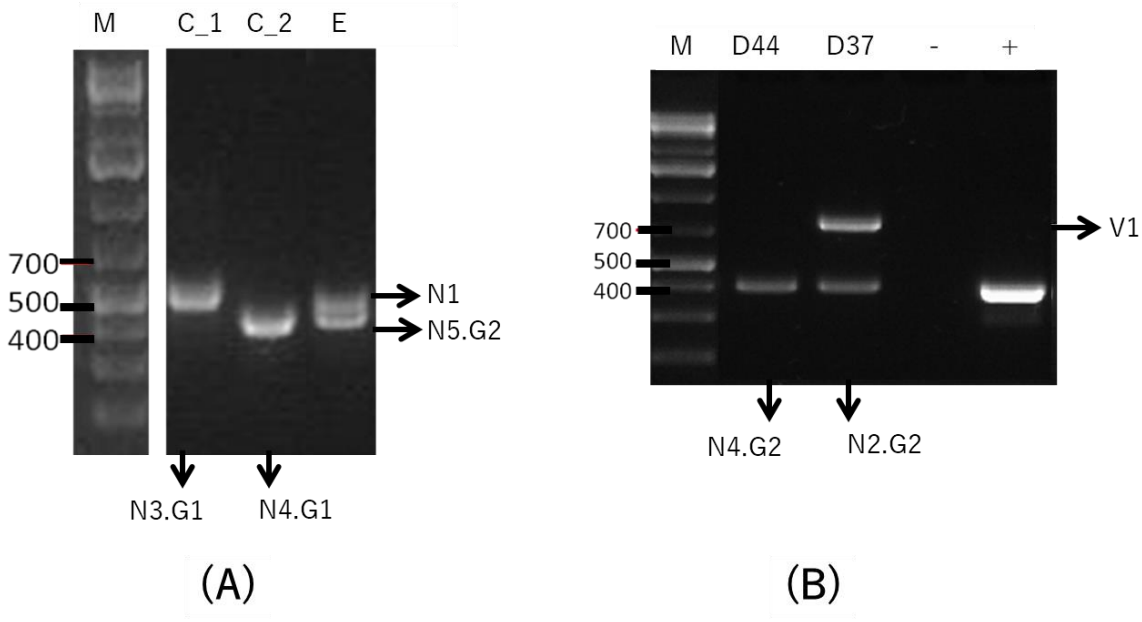


Figure 2: Light microscopy image of *Naegleria* cells on NNA plates from the sampling site D cultivated at 26°C (A), 37°C (B) and 44°C (C). Arrows indicate trophozoites (T) and cysts (C) cells observed with an inverted microscope (Nikon TS 100), x200 magnification.

The appearance of trophozoites and cystic forms was regularly inspected until the cultures reached 14 days outgrowth and the correspondence between culture findings (Figure 2) and its respective species was addressed by the molecular approach, detailed next.

2. 3. PCR amplification and sequence analysis

By using Ng.spp_FW and Ng.spp_RV primers, the PCR products obtained ranged between 395bp to 502bp whether corresponding to the expected size of a *Naegleria* species amplicon with codes from N1 to N5 and to about 750bp whether consistent with the expected fragment size of a Vahlkampfia species, with code V1, as shown in Figure 3.



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 December 2019 doi:10.20944/preprints201912.0038.v1

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Figure 3: *Naegleria* targeting PCR products analyzed on a 2% agarose gel. (A) PCR amplifications obtained from DNA extracted directly from the water sample, and (B) with the DNA extracted from cultured trophozoites. Each code in the black arrows refers to *Naegleria* (N1-N5) or *Vahlkampfia* (V1) genes. The positive control (+) was performed by using the DNA of *Naegleria gruberi* ATCC 30224 and the negative control (-) with DNA-free water. (M) refers to the marker and (C-E) indicates the sampling sites. C_1 and C_2 are upper and lower bands extracted from a previous gel, both corresponding to C sampling site.

The amplicons (Figure 3) were isolated from the agarose gel and sequenced and the codes were used to correlate the sequences we have obtained after sequencing with each corresponding species through BLASTn searches. The Table 1 summarizes the results for all the sampling sites.

Table 1: *Naegleria* and *Vahlkampfia* isolates from NNA cultures (1) and directly from the water (2) through sampling sites of the Monjolinho River (A-E), São Carlos - SP.

		1-Isolates from NNA cultures		2-Isolates directly from water samples	
		Code	**BLASTn result / Accession[1]**	**Code**	**BLASTn result/Accession[1]**
A	26 ºC	N2.G2	*N. philippinensis/ LC191904.1*	N1	*N. canariensis/FJ475124.1*
	37 ºC	N1	*N. canariensis/ FJ475124.1*		
B	26 ºC	N1	*N. canariensis/ FJ475124.1*	N2.G1	*N. philippinensis;*
				N1	*N. canariensis/ FJ475124.1*
C	26 ºC	N1	*N. canariensis/ FJ475124.1*	N4.G1	*N. australiensis*/AB128053.1;
	37 ºC	N1	*N. canariensis/ FJ475124.1*		
				N3.G1	*N. dobsoni/ KU380484.1*
				N3.G2	*N. dobsoni/ KU380484.1*
D	26 ºC	N4.G2	*N. australiensis / AB128052.1*	N2.G1	*N. philippinensis*/AY033618.1;
	37 ºC	N2.G2	*N. philippinensis/ LC191904.1*	N5.G1	*N. gruberi* / MG699123.1
		V1	*Vahlkampfia*/AB330069.1		
	44 ºC	N4.G1	*N. australiensis/* AB128053.1		
E	26 ºC	N5.G2	*N. gruberi/* MG699123.1	N5.G2	*N. gruberi*/MG699123.1;
	37 ºC	N1	*N. canariensis/ FJ475124.1*		
				N1	*N. canariensis/ FJ475124.1*

[1]Gene-Bank accession numbers.

Our findings were 95-100% identical with GenBank *Naegleria spp*. reference sequences with a query coverage of 98-100% at BLASTn alignment and the variants ".G1" and ".G2" means differences detected on the nucleotide composition within the same species. However, this internal

variability on the rDNA sequences detected within the *Naegleria* species DNA was not significant enough to originate new species.

The sequencing results from NNA plate samples (Table 1.1) revealed high identity with five distinct *Naegleria* species being *N. philippinensis, N. canariensis*, *N. australiensis* and *N. gruberi*, besides one *Vahlkampfia* gene. According to D site findings, *N. australiensis* was capable of growing at 44 ºC. Additionally, *N. dobsoni* could be isolated taking into account the identifications directly from water (Table 1-2).

2. 4. Phylogeny

To obtain the phylogenetic tree that best depicts the evolutionary relationship within the species found in this study it was built a condensed tree based on Maximum Likelihood (ML) analysis model whose statistical analysis was performed with Kimura-2 parameter model and the phylogeny was tested through 1000 bootstrap replications (Figure 4).

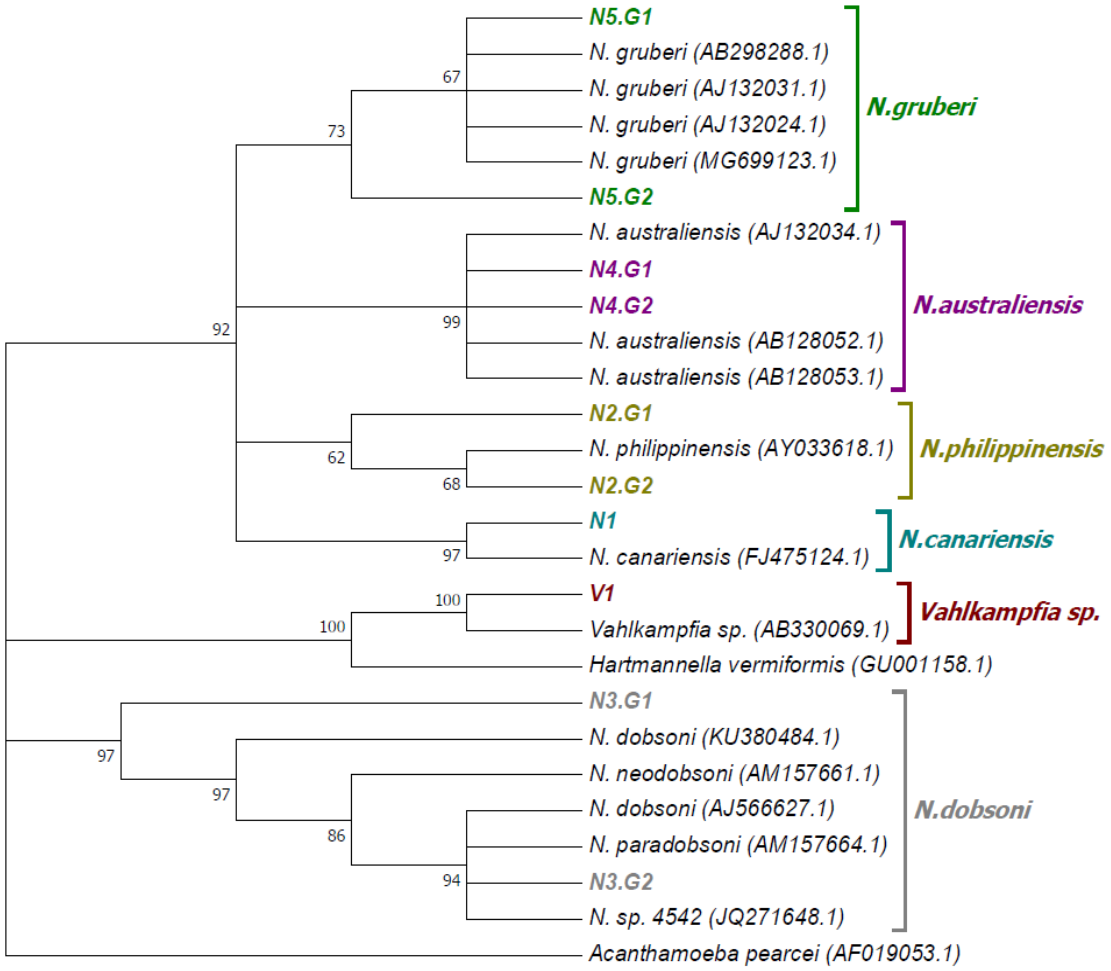

Figure 4: Evolutionary relationship among *Naegleria spp* and *Vahlkampfia spp.* based on internal transcribed sequences (ITS1 and ITS2 regions) and 5.8S sequence. *Hartmannella vermiformis* and *Acanthamoeba pearcei* were used as outgroups for *Vahlkampfia* clade and for the remaining *Naegleria* clades. The tree was condensed to present only bootstrap values higher than 50%.

The Maximum Likelihood (ML) three of the aligned ITS and 5.8S rDNA sequences clearly show the N5 (G1 and G2 variants) branching with the *Naegleria gruberi* species. N4 sequence belonging to the *N. australiensis* group, N2 to *N. philipinensis*, N1 to *N. canariensis* and N3 to the *N. dobsoni* clades. The sequence resulting from the 758bp amplicon (V1) was identified as belonging to the *Vahlkampfia*/*Hartmannella* clade. These placements in the phylogenetic tree are well supported by bootstrap replications, although the short length of the ITS-5.8S sequences does not resolve the polytomy within each species.

3. Discussion

3.1 Eutrophication along the Monjolinho River

The samples were collected along the Monjolinho river that passes through the city of São Carlos, located in the São Paulo state, Brazil. Five sampling sites were selected. The site A represents the river headwaters and the catchment area [36]. The subsequent sampling sites are located within (B and C) and nearby (D) the urban area where the population can use the water for agricultural, domestic or recreational purposes. According the limnological measurements shown in Figure 2 the dissolved oxygen and conductivity results revealed the degree of eutrophication of the Monjolinho River basin, as known parameters to evaluate levels of organic material contaminating the water [39]. From the headwater towards the two subsequent sampling sites there is a trend contributing to the gradual increase the conductivity values together with the decrease of dissolved oxygen. However, the severe reduction in electrical conductivity of the D site to the E site is likely due to the sewage treatment plant located between D and E areas. The increment on electrical conductivity in the water is closely related to organic matter decomposition, anthropogenic activities and soil flushing, as these activities elevates potassium, magnesium, calcium, carbonates and sulphates concentrations [40]. The lowest OD values found in D and E sampling sites are above the recommendations from the Brazilian Council of Environmental Regulations that determines that OD values should not be lower than 6.0 mg.L-1, as an environmental parameter of water quality [41].

The slight increase of the temperature from headwater to the last meander is likely due to the daily temperature change once the sampling followed the longitudinal course of the River (A-E) and it took an interval of five hours from the first to the last site. The gentle increase in pH value is probably consequence of the presence of alkaline compounds, such as carbonates and calcium, that can increase the pH and are also responsible for the increase of the electrical conductivity. Additionally, detergents commonly encountered in domestic wastewater have the potential to increase the pH consistent with the recorded values compared to the upstream sampling sites [40]. Nevertheless, all physical and chemical features registered in the Monjolinho River could harbor *Naegleria* as described in this paper.

3.2. Presence of Naegleria thermophilic species in Brazil

Through cultivation, particularly the trophozoites growth observed at 44°C, indicate the presence of species with pathogenic potential, as it has been discussed in earlier studies that all pathogenic species of *Naegleria* have the capability of tolerating temperature of up to 40°C [16]. The observed *N. australiensis* capability of growing and sustaining its growth at 44°C broadens the thermotolerance so far associated with this species, since none of the earlier studies have demonstrated its tolerance to 44°C neither from environmental samples nor at laboratorial conditions [4]. To date, the highest temperatures tolerated by *N. australiensis* were in the rage of 40 - 43°C [2,11,43–45].

On the other hand, *N. phillippinensis* that have been reported to grow at 40°C [4] presented consistent with our results that have confirmed its inability of withstanding 44°C. The identification of *N. australiensis* and *N. philippinensis* (Table 1) brings an important issue to be explored due to its capability of causing encephalitis [46,47] and their presence in the river water represents a threat not only to the icthyofauna but also to domestic mamalls that use the river as water supply. Moreover, although the causative agent of PAM in human, *N. fowleri*, has not been recovered in this collection, it does not exclude the need of frequent evaluating of this and other river streams. These assessment become urgent with the rising temperatures experienced due to global warming effects, [11,28,48].

3.3. Naegleria spp diversity in Brazil

Besides the identification of *N. australiensis* and *N. philippinensis*, this study also identified *N. canariensis* , *N. gruberi* and a *Vahlkampfia spp* through the same morphological approach combined with sequencing analysis. The potential of the selected primers to amplify two distinct free-living amoeba genera, *Naegleria* and *Vahlkahmpfia*, has been recently reported in two studies one from the Nile River,

Egypt [49] and the other from regions of Malaysia [50]. Taking into account the isolation of *Vahlkampfia lobospinosa* from cattle feces and the use of the Monjolinho river by domestic animals, our finding is consistent with the presence of free living amoeba, and potentially pathogenic species [51]. By analyzing DNA extracted directly from the river water (Table 1-2), one additional *Naegleria* species could be identified: *N. dobsoni*. Similarly some species were not isolated in the direct approach but after enriching the trophozoites presence by culturing we could obtain them. Thus, we highlight the importance on performing both complementary PCR analyses, using the DNA extracted directly from the river and the DNA after culturing.

To the best of our knowledge this is the first report of *N. philippineneis*, *N australiensis*, *N dobsoni* and *N. gruberi* in South America environmental samples. To date, *Naegleria* reports from South America countries have relied on *N. fowleri* identification aimed on diagnosis of samples obtained from PAM suspect victims. For instance, two Venezuelan [52] and five Brazilian [29] PAM cases have been registered. Regarding environmental isolations in this area, it has been conducted generally at genus level without assessing the diversity at the species level, as reports of *Naegleria* presence in public swimming pools of Santiago, Chile [53]. Thus, our molecular findings compose a large advance to broaden the knowledge on *Naegleria* diversity at environmental samples, mainly in the Brazilian scenario due its lack of FLA information.

4. Materials and Methods

4.1. Description of the geographical area and sample collection

Different levels of human intervention along the Monjolinho River basin were considered when selecting the five sampling sites described in the present study. To insert each sampling site (UTM coordinates) in the Monjolinho River basin image, a Geographic Information System (GIS) named Georreferenced Information Processing System (SPRING 5.5.5) was used. The resulting cadastral map (Figure 5) reveal the basin limit and the respective geographic positions of each sampling site along the main stream of the Monjolinho as follows: headwater (A: 22° 0' 2.87'' S, 47° 50' 8.96'' W), peri-urban stream (B: 21° 58' 40.6884'' S, 47° 52' 25.50'' W), urban stream (C: 21° 59' 45.40'' S, 47° 54' 6.32'' W), domestic sewage discharge (D: 22° 0' 52.41'' S, 47° 55' 26.65'' W) and after a sewage treatment plant (E: 22° 1' 34.92'' S, 47° 57' 26.29'' W). The samples were collected on Oct/2017, dry season, using one liter sterile glass flasks, in a range of 10-15 cm of the water surface, following the literature recommendation [54].

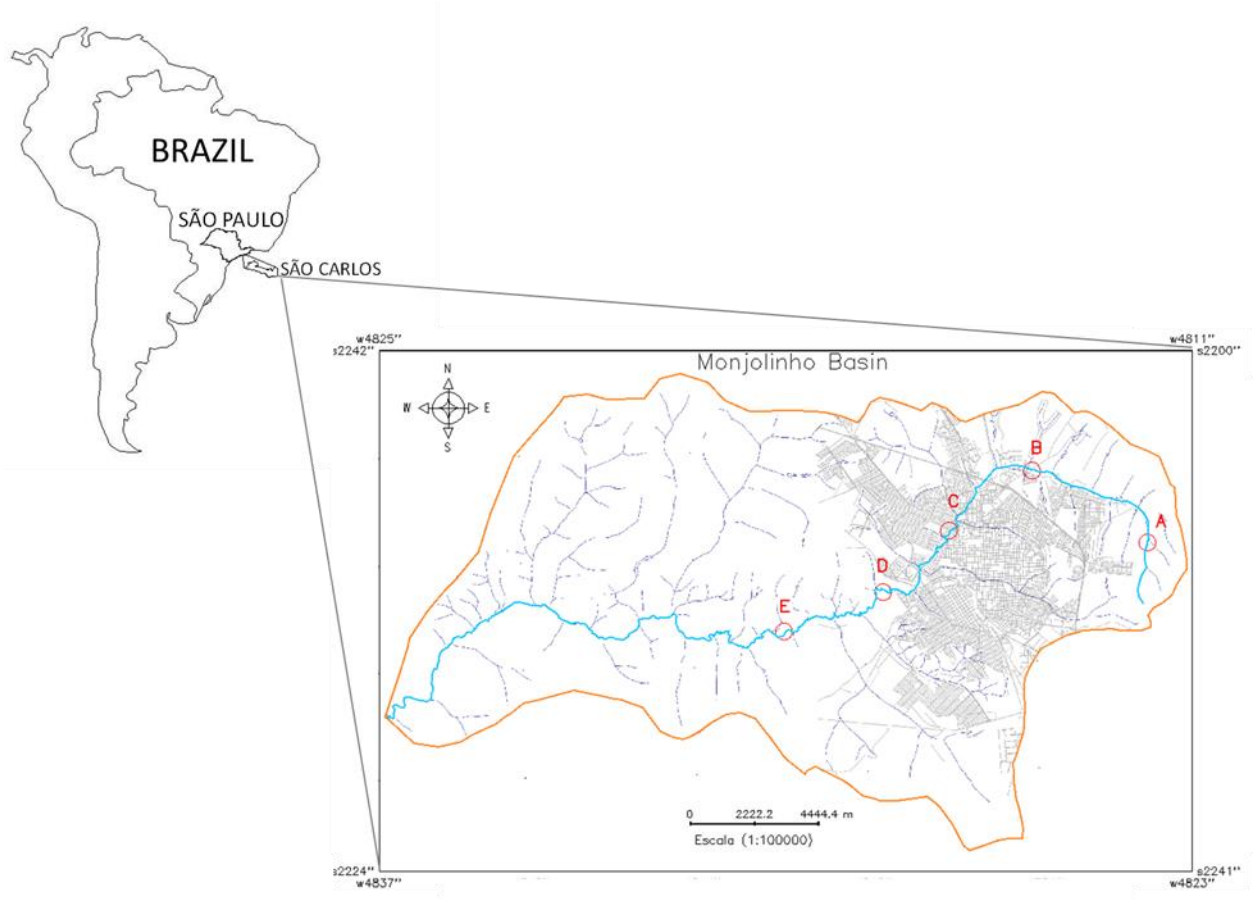

Figure 5: Cadastral map of the Monjolinho River Basin, São Carlos –São Paulo (Brazil), showing the sampling sites investigated in this study.

4.2. Limnology and sampling process

Both Global Positioning System (GPS) and liminological analysis such as dissolved oxygen (DO), hydrogenionic potential (pH) and temperature, were recorded using a portable water quality analyzer (Horiba U-10). Within 12 hours after collection the samples were homogenized and filtered through a granulometric sieve (BERTEL - ABNT/ASTM 120). 500 ml of the sieved samples were used for culturing and the remaining 500 ml for PCR amplification of target DNA and sequencing.

4. 3. Amoeba isolation and culturing

The water samples were filtered in a 0.45 µm cellulose nitrate membrane (Nalgene®) and the membranes were transferred onto 2% agar NNA plates (non-nutrient agar) [55] overlaid with heat inactivated *Escherichia coli* (60°C for 30 minutes) [47]. The plates were sealed and incubated at 26°C, 37°C and 44°C which the highest temperature has been applied to discriminate thermotolerant species [50,56].

4. 4. Morphological characterization

By daily examination of the amoeba growth, the trophozoites were sub cultured to new NNA plates to reduce the presence of contaminants and the growth was inspected in an inverted Nikon Eclipse TS 100 microscope. To infer the taxonomic identity based on the PAGE´s classification Key, the photomicrographs were screened for amoeba related morphological characters [38]. The trophozoites were harvested in 2 ml Page´s saline solution (PAS) [55], centrifuged (800xg, 10 min)

and then suspended in freezing solution (9:1, heat inactivated fetal bovine serum:DMSO) and stored in liquid nitrogen vapour.

4. 5. DNA extraction, amplification and sequencing

To perform the molecular identification directly from the water samples, 500 ml of filtered water was centrifuged at 3000 xg for 15 min at room temperature and washed once in phosphate-buffered saline (PBS). The final pellet was used for DNA extraction. Likewise, the NNA cultured trophozoites were washed once with 5 ml PAS on the agar surface to homogenization in a plate shaker (60 rpm, 10 min). The suspended cells were poured into a canonical 15 ml tube and centrifuged at 1000 xg, 10 min, RT and the pellet was solubilized with 50 µl ultrapure water (MiliQ) prior to DNA extraction. The commercial extraction kit DNeasyPowerSoil® Kit (QIAGEN) was used to achieve high DNA purity and yield for both input material – water samples and NNA cultured trophozoites. The DNA concentration was quantified in a Nanodrop 2000 Spectrophotometer (Thermo Scientific), analyzed by Agarose-Gel electrophoresis, and amplified by the polymerase chain reaction (PCR).

The DNA was amplified with *Naegleria* genus-specific oligodesoxirribonucleotides, Ng.spp_FW 5′-GAACCTGCGTAGGGATCATTT-3′ and Ng.spp_RV 5′-TTTCTTTTCCTCCCCTTATTA-3′, to the internal transcribed spacer regions (ITS1 and 2) that comprise the 5.8S rDNA gene previously adopted in phylogenetic studies [43,49,50,56,57]. The PCR reactions contained Taq High Fidelity Pol Master Mix 2x (Red, Cellco Biotec), 100 ng of the DNA template and 200 nmoles of each primer. The reactions were incubated in a T100 Thermal Cycler (BIO-RAD) under a cycling condition of 95 °C for 3 min followed by 35 cycles at 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 2 min. Amplified fragments were examined in SYBR Safe stained 2% agarose gels. The amplified DNA bands were purified using the Agarose Gel Extraction Kit (Cellco Biotec), cloned into pJET 1.2/blunt cloning vector (Thermo-Scientific) and transformed into *E. coli* TOP 10 competent cells. The plasmid DNA was isolated with Fast-n-Easy Plasmid Mini-Prep Kit (Cellco Biotec) and sequenced in triplicate in both directions in a 3130 Genetic Analyzer (Thermo Scientific), therefore each base was independently sequenced six times. The chromatograms were carefully examined with DNA analysis software (SnapGene Viewer) and the nucleotide homology was investigated through Basic Local Alignment Search Tool (BLAST) at National Centre for Biotechnology Information (NCBI) blasting with Eukarya Domain sequences.

4. 6. Phylogenetic inferences

Phylogenetic analysis was based on the ITS and 5.8S sequences from this work. The consensus sequences were aligned with the *Naegleria* rDNA reference sequences already deposited in the GenBank database, using ClustalW software with the following gap penalties: 20/0.1 and 15/6.66 to gap opening/extension for pairwise and multiple alignments, respectively. The final alignment was used for the phylogenetic analysis with the Molecular Evolutionary Genetics Analysis (MEGA7) to build the phylogenetic tree based on maximum likelihood (ML) inference method with Tamura-Nei statistic model [58]. 1000 bootstrap replicates were performed to determine the statistical reliability of each node.

5. Conclusions

In this study we describe the identification of *Naegleria* along the Monjolinho River Basin with morphological and molecular approaches. Considering the fact that earlier Brazilian studies have focused the *Naegleria* environmental investigations based on morphological analysis they have reported the information at genera level. In this context, the present research was the first Brazilian study to perform a molecular approach to detect *Naegleria* in an environmental sample and in which the findings could change the scenario by revealing its presence at the species level. Thus, it increases the knowledge on diversity due the identification of five distinct *Naegleria* species and one

Vahlkampfia gene. The limnological characterization accomplished by this study has allowed the assessment of eutrophication, whose D sampling site was pointed as the most eutrophic compared to the remaining four sites. Additionally, it was the most diverse regarding species identified as it revealed three of the five *Naegleria spp* and the *Vahlkampfia* occurrence along the river. Although *N. fowleri* has not been recovered in this survey, the presence of *N. australiensis* and *N. phillipinensis* distributed from the headwater to the fourth sampling site indicates that potentially pathogenic species could grow at Monjolinho River. Even if they have never been reported in human infections, its potential to cause encephalitis in laboratory conditions renders these species worth a more careful investigation. Since both species were found in the most eutrophic sampling site, there is a concern on investigating surrounding freshwater systems with similar limnological conditions that could favor its presence and dispersion. Our findings have strengthened the importance of simultaneously combining morphological approaches with molecular investigation aiming to reach a deeper investigation of *Naegleria spp*. in an environmental sample. This study highlights the need to perform further investigations addressing the presence and distribution of potentially pathogenic FLA genera in Brazil.

Funding: NKB was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES) fellowship, This work was funded by BIOTA São Paulo Research Foundation (FAPESP) program, #2018/20693-4 to OHT.

Acknowledgments: We would like to thank the members of the Structural Biology Laboratory (IFSC-USP) for helpful discussions in the course of this work. We thank specially to José Valdecir de Lucca for the assistance in the water sample collection. We would like to thank also Dr. Susana Andrea Sculaccio Beozzo for technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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