Presence of potentially pathogenic free-living amoebae strains from well water samples in Guinea-Bissau

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Free-living amoebae (FLA) include opportunistic pathogens such as *Naegleria fowleri*, *Balamuthia mandrillaris*, and the genera *Sappinia* and *Acanthamoeba*. In this study, a survey was conducted in order to evaluate the presence of potentially pathogenic amoebic strains in water samples collected from wells located in the western part of Guinea-Bissau. The samples were left to precipitate for 48 hours and then the sediments were seeded on non-nutrient agar plates containing *Escherichia coli* spread and cultures were checked daily for the presence of FLA. Identification of FLA strains was based on the morphological and polymerase chain reaction (PCR) using the 18S rDNA or 16S mitochondrial rDNA genes in the case of *Naegleria* and *Balamuthia* genera, respectively. In the case of positive samples of *Acanthamoeba*, strains were further classified at the genotype level by sequencing the diagnostic fragment 3 (DF3) region located in the 18S rDNA gene as previously described. *Sappinia* sp. was not isolated during the study and thus, no molecular analysis was performed for this genus. The obtained results revealed the presence of *Acanthamoeba* (genotypes T3 and T4), *Naegleria fowleri*, and *Balamuthia mandrillaris*. To the best of our knowledge, this is the first report demonstrating the presence of FLA in water bodies from Guinea-Bissau and the first report on the isolation of *Balamuthia mandrillaris* from environmental sources in Africa.

Keywords: *Acanthamoeba*, *Balamuthia mandrillaris*, *Naegleria*, Well water, Genotype, Guinea-Bissau

Introduction

Free-living amoebae (FLA) are widely distributed protozoa in the environment that are able to survive in different habitats such as water, soil, dust and air sources.[1,2] Among FLA, some species are causative agents of disease in humans and other animals, such as *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*.[1,3] Specifically, *N. fowleri*, *B. mandrillaris*, and *S. diploidea* have been reported as causative agents of encephalitis. Moreover, *Acanthamoeba* pathogenic strains are the causative agents of a multifocal encephalitis called granulomatous amebic encephalitis, a chronic central nervous system disease of immunocompromised hosts, and various other system disease states, including *Acanthamoeba* keratitis (AK) and pneumonitis.[2] Most studies have shown that 90% of *Acanthamoeba* isolates that produce infections belong to the T4 genotype, although other genotypes have been reported as causative agents of amoebic infections in humans and other animals, such as T1, T3, T5, T10, T11, T15, T17, and T18.[2,4]

The presence of FLA in water bodies may represent a health risk for both immunocompromised and immunocompetent individuals. Moreover, FLA are highly resistant to extreme conditions of temperature, pH, and exposure to chemicals. In addition to their pathogenicity, FLA serve as hosts for a large number of pathogenic bacteria and viruses of clinical relevance for humans and other animals.[2]

It is important to mention that there is a lack of studies in the African continent about the distribution
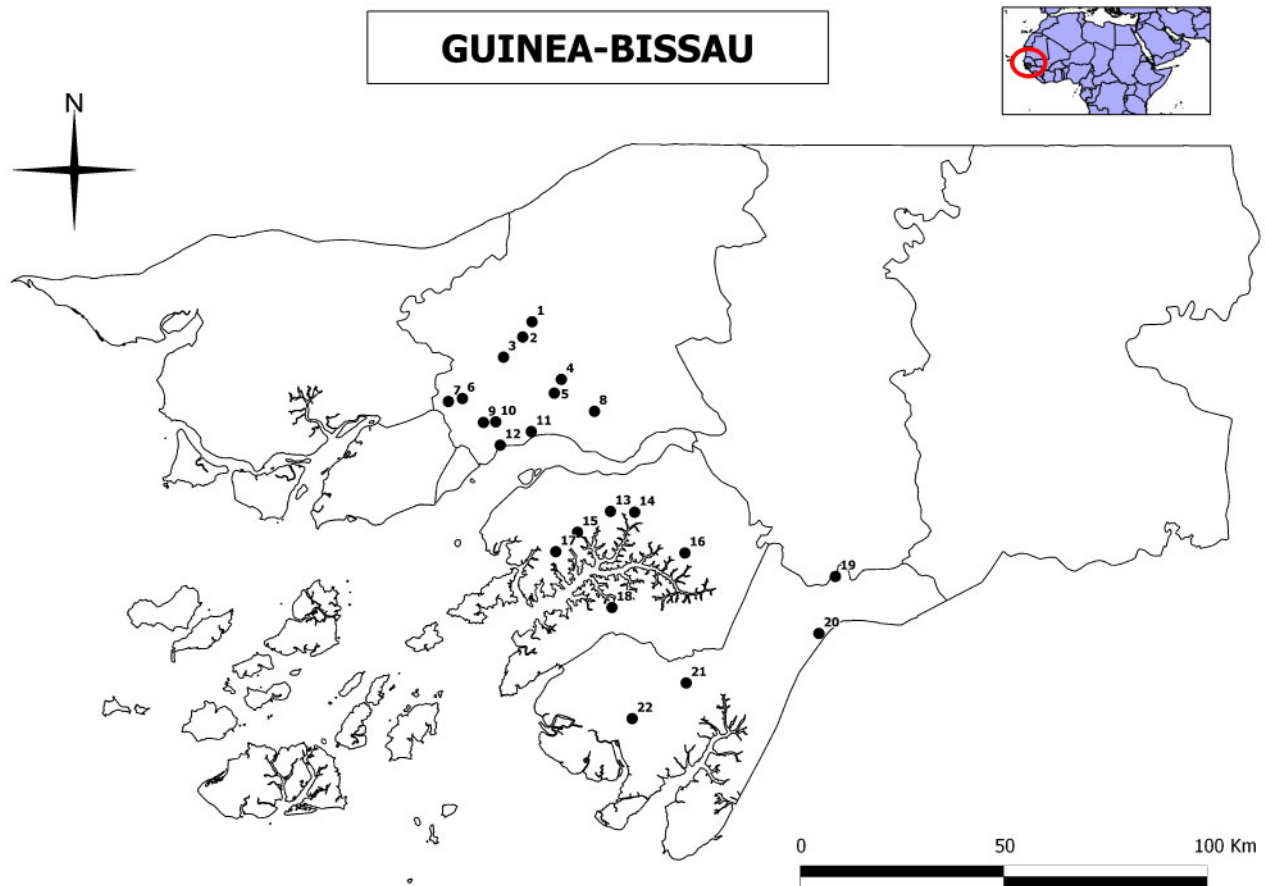
Figure 1 Sampling points surveyed during 2011 in three regions of Guinea-Bissau. Oio Region: 1, Culcunhe; 2, Watine; 3, Ufle; 4, Braia; 5, Clack N'djassé; 6, Bera; 7, Palche Iala; 8, Wedequeia; 9, Saw; 10, Djugudul Com; 11, Thugue; 12, Oco Grande. Quínara Region: 13, Ganjetra; 14, Fulacunda; 15, Brandao; 16, Madina Hatche; 17, Nova Sintra; 18, Empada. Tombalí Region: 19, Madina Contabane; 20, Afia Bunhio; 21, Botchi Minde; 22, Mato Forroba.

of FLA in environmental sources, such as soil and water bodies. Nevertheless, studies on the presence of potentially pathogenic genotypes of *Acanthamoeba*, as well as *Naegleria fowleri*, in environmental sources has been reported by other authors in this continent (including countries such as Nigeria, Tunisia, Benin, and Egypt among others).[5–9] Moreover, only a few clinical cases of amoebic encephalitis have been reported in Nigeria (*N. fowleri*),[10–12] South Africa (*N. fowleri*),[13] and Senegal (*Acanthamoeba*).[14] Furthermore, up to five cases of AK have been informed in northern Africa (Tunisia).[15,16] Up to date, *Sappinia* spp. or *B. mandrillaris* have not been reported in Africa. Nevertheless, the presence of *B. mandrillaris* in this continent is suspected since immunoreactivity towards this species in individuals from Ivory Coast was recently reported.[17]

Therefore, the aim of this study was to determine the presence of potentially pathogenic FLA in water samples from Guinea-Bissau. To the best of our knowledge, this is the first study of *Acanthamoeba* strains at the genotype level in this country and the first report on the isolation of *Balamuthia mandrillaris* from environmental sources in Africa.

Materials and Methods
Sample sites and culture of FLA
Twenty-two water samples were collected from wells during the wet season of 2011 at rural areas from three regions of the western part of Guinea-Bissau (Fig. 1). The two southern regions (Quínara and Tombalí) are undisturbed forest areas, whereas the northern one (Oio) has slightly human impacts by extensive agricultural and livestock uses. Water samples were collected from the wells most frequently used by the local population in 22 *tabankas* (small traditional villages). Average depth of wells was always less than 20 m. Values of pH, conductivity (µS/cm), and total hardness (mg/l CaCO$_3$) of the collected water samples were also determined at collection. Conductivity and pH were measured *in situ* with a HANNA Model HI9829 portable conductivity/pH meter. Total hardness was determined with a Lovibond PC MultiDirect spectrophotometer. Three water replicates of 1 l were collected in each well. Samples were left 48 hours at room temperature so that precipitation could occur. After that, the sediment was carefully removed and transferred to 50 ml sterile polypropylene tubes.

After that, 150 µl of each sediment sample were seeded onto 2% non-nutrient agar plates with heat killed *E. coli* and incubated at 25°C. The cultures were monitored every 24 hours, for up to 7 days. Positive isolates for FLA (identified under the inverted microscope) were cloned by dilution and transferred to new culture plates for further molecular analyses as previously described.[7] In the case of positive samples for *Acanthamoeba*, amoebae were transferred into axenic cultures by placing them in PYG medium [0.75% Proteose peptone (w/v), 0.75% yeast extract (w/v), and 1.5% glucose (w/v)]. A type strain of *Acanthamoeba* (*Acanthamoeba castellanii* Neff ATCC 30010) from the American Type Culture Collection (ATCC) was grown without shaking under the same conditions. Other genera of FLA were not axenified.

DNA extraction

DNA from cultures identified as positive for FLA by microscopy was extracted by placing 1–2 ml of amoebic cultures directly into the Maxwell® 16 Tissue DNA Purification Kit sample cartridge (Promega, Madrid, Spain). Amoebic genomic DNA was purified using the Maxwell 16 Instrument as described in the Maxwell 16 DNA Purification Kits Technical Manual #TM284 (Promega). DNA yield and purity were determined using the NanoDrop® 1000 spectrophotometer (Fisher Scientific, Madrid, Spain).

Polymerase chain reaction (PCR) reactions and molecular characterization of FLA isolates

For the identification and genotyping of *Acanthamoeba* isolates, the diagnostic fragment 3 (DF3) region of *Acanthamoeba* 18S rDNA gene was amplified as previously described using the JDP1/JDP2 primer pair.[18,19] This PCR amplifies a 495 bp fragment of the 18S rDNA gene that allows genotype identification after sequencing.

For Vahlkampfiidae of the genus *Naegleria*, the complete ITS region (ITS1, 5.8S, and ITS2) was amplified using the NFITSFW and NFITSRV primer pair for *N. fowleri* and the ITSFW and ITSRV primers for other *Naegleria* species as previously described,[20,21] which amplify a fragment of around 300–400 bp depending on the species of *Naegleria*. In the case of other Vahlkamphfiidae, the JITSFW/JITSRV primer pair was used as previously described.[21] Moreover, the 18S rDNA gene of these FLA was also amplified using the universal eukaryotic P2 and P3r primer pair, which amplifies a fragment of around 500 bp in FLA and allows identification of FLA at the genus level after sequencing.[22] *Naegleria canariensis* CCAP 1518/27 DNA was used a positive control in the PCR reactions.

For the identification of *Balamuthia mandrillaris*, the 16S rDNA gene was amplified and sequenced as previously described,[19] using the Balspec16S primer pair which amplifies a product of 1075 bp. *Balamuthia mandrillaris* CDC:V416 strain (ATCC®PRA-290™) DNA was used as a positive control.

The obtained PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using a MEGABACE 1000 automatic sequencer (Healthcare Biosciences, Barcelona, Spain) in the University of La Laguna Sequencing Service (Servicio de Secuenciación SEGAI, University of La Laguna). Sequences were obtained twice from both strands. The obtained sequences were aligned using Mega 5.0 software program.[23] Moreover, nucleotide similarity search was performed by BLAST search (Basic Local Alignment search tool) of the sequenced amplicons against amoeba species.

Genotype identification of *Acanthamoeba* was carried out based on sequence analysis of DF3 region as previously described by comparison to the available *Acanthamoeba* DNA sequences in Genbank database.[18,19] *Acanthamoeba castellanii* Neff ATCC 30010 (genotype T4) DNA was used as a positive control in the PCR reactions. Phylogenetic analyses for *Acanthamoeba* strains was carried out using maximum parsimony, minimum evolution and maximum likelihood optimality criteria, implemented in Mega 5.0.[23] Transition/transversion ratios were estimated by maximum likelihood heuristic searches. Estimates of node support were obtained by performing 1000 bootstrap replicates. Obtained sequences were compared to sequences available in GenBank database.

The sequences for the new FLA isolates are deposited in the Genbank database under the accession numbers KJ000391–KJ000399 and are shown in Table 1.

Results and Discussion

Regarding the collected water samples, the mineral content was highly variable within the studied area, with pH values ranging from 2.12 to 6.00, and conductivity values varying between 43 and 524 µS/cm. Furthermore, total hardness varied between 7 and 350 mg/l (Table 1). However, no correlation could be found between the concentration of the different parameters and the presence of potentially pathogenic FLA. Mainly because of the high resistance of FLA against harsh environmental conditions as it has been reported before.[2]

FLA were isolated in 15 of the 22 samples (68.2%) included in this study (Table 1). However, *Sappinia* spp. was not identified within these samples. Members of the Vahlkampfidae family were isolated in 13 of the 15 positive samples (86.6%). However, most of the isolated strains belonged to *Willaertia* or *Tetramitus* genera (10 samples), which are non-pathogenic FLA and are widely distributed world-

wide in water related habitats. Moreover, *Naegleria* genus was also observed in five of the 13 samples that were Vahlkampfidae-positive. Interestingly, sequencing of the FLA 18S rDNA gene and ITS region revealed the presence of *Naegleria fowleri* in four of the five positive samples for the genus *Naegleria* (98–99% homology with other *N. fowleri* strains available in Genbank). Previous studies have reported the isolation of *N. fowleri* from dust and soil from Nigeria[5,12] and water bodies in Egypt.[6] Interestingly, human cases of encephalitis due to *N. fowleri* have been reported in Nigeria (2)[10,11] and South Africa (1).[13] In all the reported cases, water and/or dust sources were suspected to be the source of infection.

Regarding *Acanthamoeba* genus, four of the 15 positive samples (27.6%) were positive for *Acanthamoeba* strains belonging to genotype T3 (one sample, 25%) and T4 (three samples, 75%) (Fig. 2). Previous environmental studies in different countries in Africa have reported the presence of potentially pathogenic *Acanthamoeba* strains mainly in water sources.[6–9] Furthermore, only three clinical related studies regarding *Acanthamoeba* genus have been reported in Africa: one case of encephalitis due to *Acanthamoeba* in Dakar, Senegal,[14] an epidemiological study to determine the presence of FLA in the nasal passages of a population in Zaria, Nigeria[24] and the report of three cases of AK in patients from Tunisia.[16] Another case of encephalitis

due to a hartmannellid amoeba was reported in Zambia;[25] however, it is possible that the identification of this strain was not carried out correctly.

Finally, one sample was positive for *B. mandrillaris*. Interestingly, the region where this water sample was collected is often flooded by high tides during the rainy season and showed high values of salinity. Nevertheless, and to the best of our knowledge, this is the first report of *B. mandrillaris* isolation from the environment in the African continent. Although this study may be the first to identify *B. mandrillaris* from the environment in Africa, it should be noted that its presence on the continent was suspected because of the prevalence of immunoreactivity towards this species in individuals in Ivory Coast (Western Africa).[17] Despite all the recent advances in the study of *B. mandrillaris*, several questions about this organism remain unanswered. Although the number of infections due to *B. mandrillaris* is relatively low, the estimated frequency of reported cases so far is likely to be underestimated.[26] However, even in the present study, environmental isolation of *B. mandrillaris* seems to be a low frequency occurrence. In addition to this study, researchers have only been able to isolate *B. mandrillaris* from the environment on five occasions. The development of improved culture techniques to allow a fast, reliable, and repetitive approach to isolate

Table 1 Free-living amoebae isolated in the analysed 22 water samples from Guinea-Bissau (Fig. 1). Values of pH, conductivity (μS/cm), and hardness (mg/l), and Genbank accession numbers for the new FLA strains are also shown

| Sampling point	Location coordinates		Isolated amoebae	pH	Conductivity	Hardness	Accession #
(1) Culcunhe	N12°14′36.3″	W15°25′30.0″	Negative	4.08	346	190	
(2) Watine	N12°12′21.7″	W15°26′52.0″	*Naegleria fowleri, Tetramitus* sp.	4.06	275	170	KJ000395
(3) Ufle	N12°09′23.3″	W15°29′42.1″	Negative	4.15	355	90	
(4) Braia	N12°06′06.4″	W15°21′10.5″	Negative	2.20	43	90	
(5) Clack N'djassé	N12°04′05.0″	W15°22′12.9″	*Acanthamoeba* sp. T4 (GBW-1), *Willaertia* sp.	2.25	392	230	KJ000391
(6) Bera	N12°03′17.4″	W15°35′45.7″	*Willaertia* sp.	4.17	306	200	
(7) Palche Iala	N12°02′50.7″	W15°37′50.0″	*Naegleria fowleri, Tetramitus* sp.	4.70	213	90	KJ000396
(8) Wedequeia	N12°01′23.9″	W15°16′17.3″	Negative	2.23	…	350	
(9) Saw	N11°59′45.3″	W15°32′40.0″	*Willaertia* sp.	2.14	102	80	
(10) Djugudul Com	N11°59′50.8″	W15°30′51.1″	Negative	2.12	197	110	
(11) Thugue	N11°58′ 25.1″	W15°25′36.8″	*Tetramitus* sp.	2.16	216	160	
(12) Oco Grande	N11°56′24.4″	W15°30′11.2″	*Tetramitus* sp.	2.22	524	300	
(13) Ganjetra	N11°46′38.7″	W15°13′56.0″	*Willaertia* sp.	6,00	44	15	
(14) Fulacunda	N11°46′30.9″	W15°10′21.9″	*Naegleria* sp., *Willaertia* sp.	5.10	328	96	
(15) Brandao	N11°43′34.0″	W15°18′47.5″	Negative	5.10	78	18	
(16) Madina Hatche	N11°40′30.4″	W15°02′57.3″	*Acanthamoeba* sp. T3 (GBW-4), *Willaertia* sp	5.20	49	14	KJ000394
(17) Nova Sintra	N11°40′41.8″	W15°21′59.9″	*Balamuthia mandrillaris*	5.50	100	42	KJ000399
(18) Empada	N11°32′27.9″	W15°13′43.2″	*Naegleria fowleri, Tetramitus* sp	5.10	71	7	KJ000397
(19) Madina Contabane	N11°37′02.6″	W14°40′45.8″	*Acanthamoeba* sp. T4 (GBW-2), *Willaertia* sp	5.50	111	10	KJ000392
(20) Afia Bunhio	N11°28′38.2″	W14°43′11.8″	*Acanthamoeba* sp. T4 (GBW-3)	6.10	241	150	KJ000393
(21) Botchi Minde	N11°17′05.6″	W15°02′23.6″	Negative	5.10	283	10	
(22) Mato Forroba	N11°16′04.7″	W15°10′43.8″	*Naegleria fowleri, Tetramitus* sp.	5.50	100	39	KJ000398
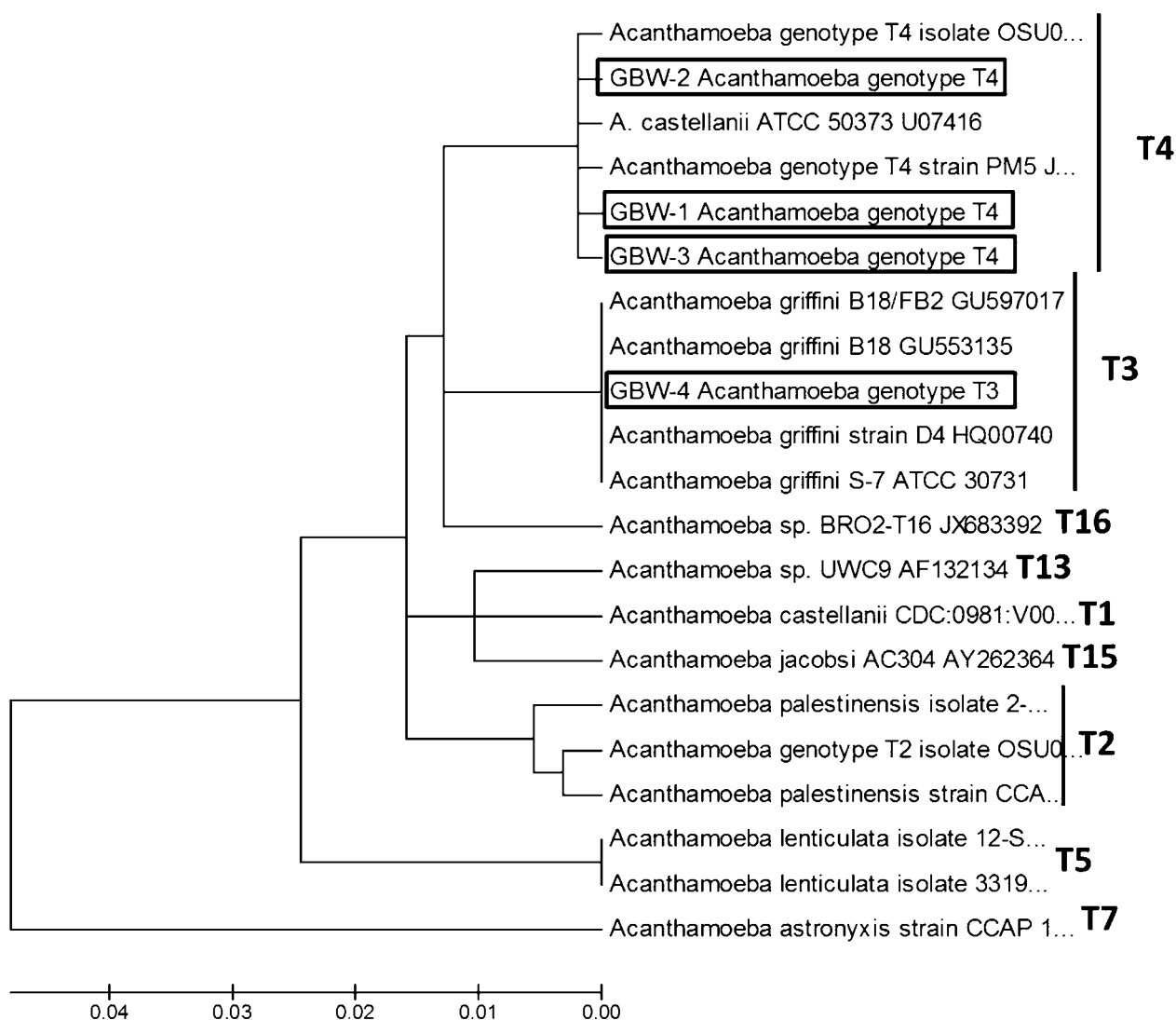
Figure 2 18S rDNA DF3 linearized neighbour-joining tree obtained by using the Kimura two-parameter distance algorithm, produced in MEGA 5.0. The isolates obtained in the present study are identified in the tree (boxes). The type sequences were taken from GenBank and are presented under the following numbers: *A. astronyxis* strain CCAP 1534/1 #AF239293, *A. castellanii* strain CDC:0981:V006 Accession #U07400, *A. castellanii* ATCC 50373 Accession # U07416, *A. griffini* S-7 ATCC 30731 Accession #U07412, *A. griffini* B-18 FB2 Acession #GU597017, *A. griffini* B-18 Acession #GU553135, *A. griffini* strain D4 Acession #HQ00740, *A. lenticulata* isolate 12-SO #KC694184, *A. lenticulata* isolate 33195463 Accession #KC438381, *Acanthamoeba* sp. isolate OSU09-002 Accession # JQ669657, *Acanthamoeba* sp. genotype T2 Isolate OSU09-006 #JQ669661, *Acanthamoeba palestinensis* isolate TW-2 Accession #KC694193, *A. palestinensis* strain CCAP 1547-1 #AF239296, *Acanthamoeba* sp. isolate BRO2-T16 Accession #JX683392, *Acanthamoeba* sp. UWC9 Accession #AF132134, *Acanthamoeba* sp. PM5 Accession #JX494395, *Acanthamoeba jacobsi* AC304 Accession #AY262364.

and grown these amoebae in axenic conditions is urgently required.

Nevertheless, the present study reports the presence of potentially pathogenic FLA strains of *Acanthamoeba* genus and *Naegleria fowleri* and *Balamuthia mandrillaris* species in water sources from Guinea-Bissau. The isolation of these potential pathogens from the studied water sources in this country should raise awareness within clinicians and public health professionals. Moreover, knowledge of the prevalence of FLA in this country may help clinicians to diagnose and treat either healthy or immunocompromised individuals. Presence of potentially pathogenic FLA in the studied wells should

lead to take precautions in order to avoid potential health risks to individuals inhabiting these areas.

Disclaimer Statements

Conflicts of interest The authors declare no conflicts of interest.

Ethics approval No applicable.

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