



Isolation and molecular identification of free-living amoebae from dishcloths in Tenerife, Canary Islands, Spain

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Abstract

In this work, the presence of free-living amoebae (FLA) in dishcloths collected from human activity related places was evaluated. Once in the laboratory, 6 cm² pieces of each dishcloth were cut and washed with Page's Amoeba Solution (PAS) in sterile tubes. After washing, the dishcloth pieces were removed, and the tubes were centrifuged (1500 rpm for 10 min). The obtained pellets were seeded onto 2% non-nutrient agar (NNA) plates, incubated at room temperature and were monitored daily an inverted microscope. Once clonal cultures were obtained (only one type of FLA observed), molecular analyses were carried out in order to characterize the isolated FLA strains at the genus/genotype level. From the 31 dishcloths which were processed, FLA strains were isolated in NNA plates in 13 the samples (13/31, 42%). However, and due to bacterial overgrowth, only six strains were characterized at the molecular level (PCR and sequencing). Among the PCR positive strains, 83.33% (5/6) of the PCR positive samples belonged to *Acanthamoeba* genus (80% (4/5) to genotype T4 and 20% (1/5) to genotype T11). Furthermore, one strain was identified as a member of *Allovahlkampfia* genus using both morphological and molecular approaches. To the best of our knowledge, this is the first report on the isolation of *Allovahlkampfia* genus from dishcloths and in the Spanish territory. The presence of FLA in dishcloths should raise awareness to improve hygienic strategies in food- and domestic-related environments, in order to prevent contamination with these protozoa, which are able to be pathogenic and even to act as vehicles of other pathogenic agents.

Keywords *Acanthamoeba* · *Allovahlkampfia* · PCR · Dishcloths · Canary Islands · Spain

Introduction

Free-living amoebae (FLA) are widely distributed protozoa which are able to colonize different environments such as water, soils, dust, and air among others (Marciano-Cabral and Cabral 2003; Siddiqui and Khan 2012; Lorenzo-Morales

et al. 2013). To date, six amoebae have been identified as being able to infect humans or other animals: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, *Vahlkampfia* spp. *Sappinia pedata*, and *Vermamoeba vermiformis* (Visvesvara et al. 2007; Scheid et al. 2008; Qvarnstrom et al. 2009; Smirnov et al. 2011). Some of these infections are opportunistic, occurring mainly in immunocompromised hosts (*Acanthamoeba* and *Balamuthia* encephalitis), while others affect to immunocompetent individuals (*Acanthamoeba* keratitis, *Naegleria fowleri* meningoencephalitis and some cases of *Balamuthia* encephalitis) (Shuster and Visvesvara 2004; Visvesvara et al. 2007). Nevertheless, other FLA species have been reported as human pathogens, such as members of *Allovahlkampfia* genus, which has been reported as a causal agent of human keratitis (Tolba et al. 2016). These amoebae are not only able to act as opportunistic pathogens but also as vehicles of other pathogenic agents such as bacteria, fungi, and viruses (Thomas et al. 2010; Lasjerdi et al. 2015; Pagnier et al. 2015a, b).

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Furthermore, some bacteria species have developed strategies to avoid FLA digestion (Scheid and Schwarzenberger 2012) and are currently known as amoeba-resistant bacteria (ARB) (Greub and Raoult 2004). There are different studies which have revealed the importance of FLA in the transmission and/or survival of pathogenic bacteria in the environment such as *Legionella* spp., *Mycobacterium* spp. (Scheid 2014; Cabello-Vílchez et al. 2014), *Campylobacter jejunii* or *Vibrio*

harveyi (Reyes-Batlle et al. 2017a, b). Therefore, the presence of FLA in water and human-related fomites could represent a risk source for FLA and also ARB.

In 2014, Chavatte and colleagues carried out a study where they isolated FLA and foodborne bacterial pathogens from dishcloths collected in Belgium. In the present study, the presence of FLA was evaluated in dishcloths collected in Tenerife island, Spain which were used to

Table 1 Dishcloths evaluated in this study (m, month; w, week; d, day)

Sample code	Use	Length use	Place	Disinfectant
B1	Domestic	5–6 m	Kitchen	–
B2	Domestic	1 m	Surfaces	Detergent
B3	Professional (Supermarket)	4 m	Surfaces	Bleach
B4	Domestic	5 m	Surface	–
B5	Domestic	3 m	Wet surfaces	–
B6	Domestic	1 m	Surfaces	Detergent
B7	Domestic	5 m	Surfaces	–
B8	Domestic	7 m	Surfaces	–
B9	Professional (Supermarket)	2 w	Surfaces	–
B10	Domestic	1 w	Dust and greasy surfaces	–
B11	Professional (Coffee shop)	1 w	Surfaces	–
B12	Domestic	1 w	Floor	Floor cleaner
B13	Domestic	4 m	Toilet	Toilet detergent
B14	Professional (Coffee shop)	1 w	Surfaces	–
B15	Professional (Coffee shop)	1 w	Surfaces	–
B16	Domestic	1 m	Toilet	Floor cleaner
B17	Domestic	3 w	Windows	–
B18	Domestic	4 w	Wet surfaces	–
B19	Domestic	3 w	Surfaces	–
B20	Domestic	1 m	Surfaces	–
B21	Domestic	1 d	Surfaces	–
B22	Domestic	2 w	Surfaces	–
B23	Professional (Supermarket)	4 d	Butcher	–
B24	Domestic	2 w	Terrace surfaces	–
B25	Professional (Supermarket)	1 w	Surfaces	–
B26	Professional (Supermarket)	3 d	Surfaces	–
B27	Domestic	1 w	Windows	–
B28	Domestic	3 d	Surfaces	–
B29	University Dorm	2 w	Surfaces	–
B30	Domestic	1 m	Kitchen	Bleach
B31	Professional (Supermarket)	2 w	Surface	–

clean not only food-related areas but also other domestic zones such as windows or toilets.

Material and methods

Sampling sites and sample collection

During January and February of 2018, a total of 31 dishcloths were collected from different domestic and food-related places and were stored in sterile bags at 4 °C until further processing. Among the dishcloths included in this study, most of them were used for domestic pur-

poses although some of the samples were used in supermarkets or coffee shops (Table 1).

Once in the laboratory, 6 cm² pieces of each dishcloth were cut and washed with Page's Amoeba Solution, ATCC Medium 1323 (PAS) in sterile tubes. After this washing, the dishcloth pieces were removed and the tubes containing the wash were centrifuged (1500 rpm for 10 min). The obtained pellets were seeded onto 2% non-nutrient agar (NNA) plates, incubated at room temperature, and monitored daily under an inverted microscope. Positive plates (classified at the morphological level using Page key (Page, 1988) were cloned by dilution in NNA until a monoxenic culture was obtained as previously described (Lorenzo-Morales et al. 2005; Reyes-

Fig. 1 **a** *Acanthamoeba* spp. at × 20 of magnification isolated from some of the analyzed dishcloths. A: cyst from B8 sample; B: cysts and trophozoites from B11 sample. **b** 18S rDNA DF3 linearized neighbor-joining tree obtained by using the Kimura two-parameter distance algorithm, produced in MEGA X. The obtained isolates in the present study are identified in the tree (boxes). The type sequences were taken from GenBank database

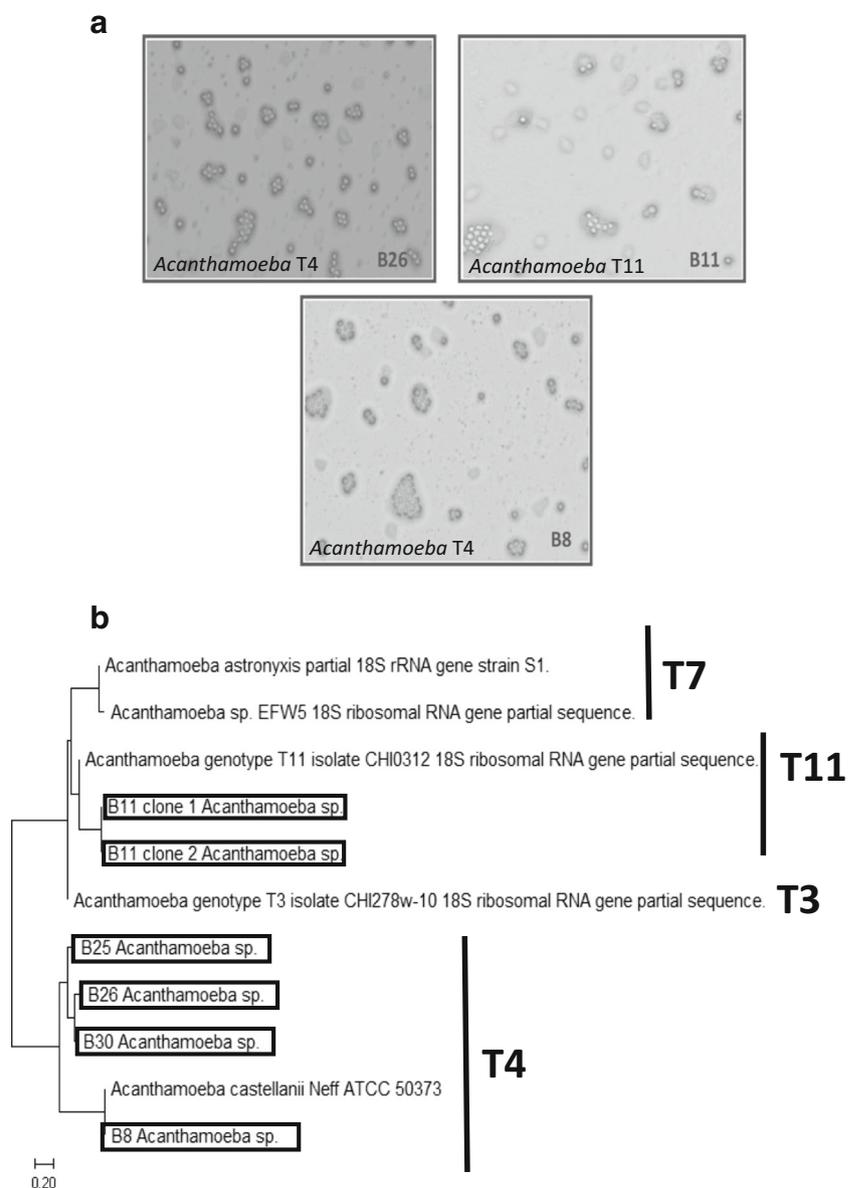
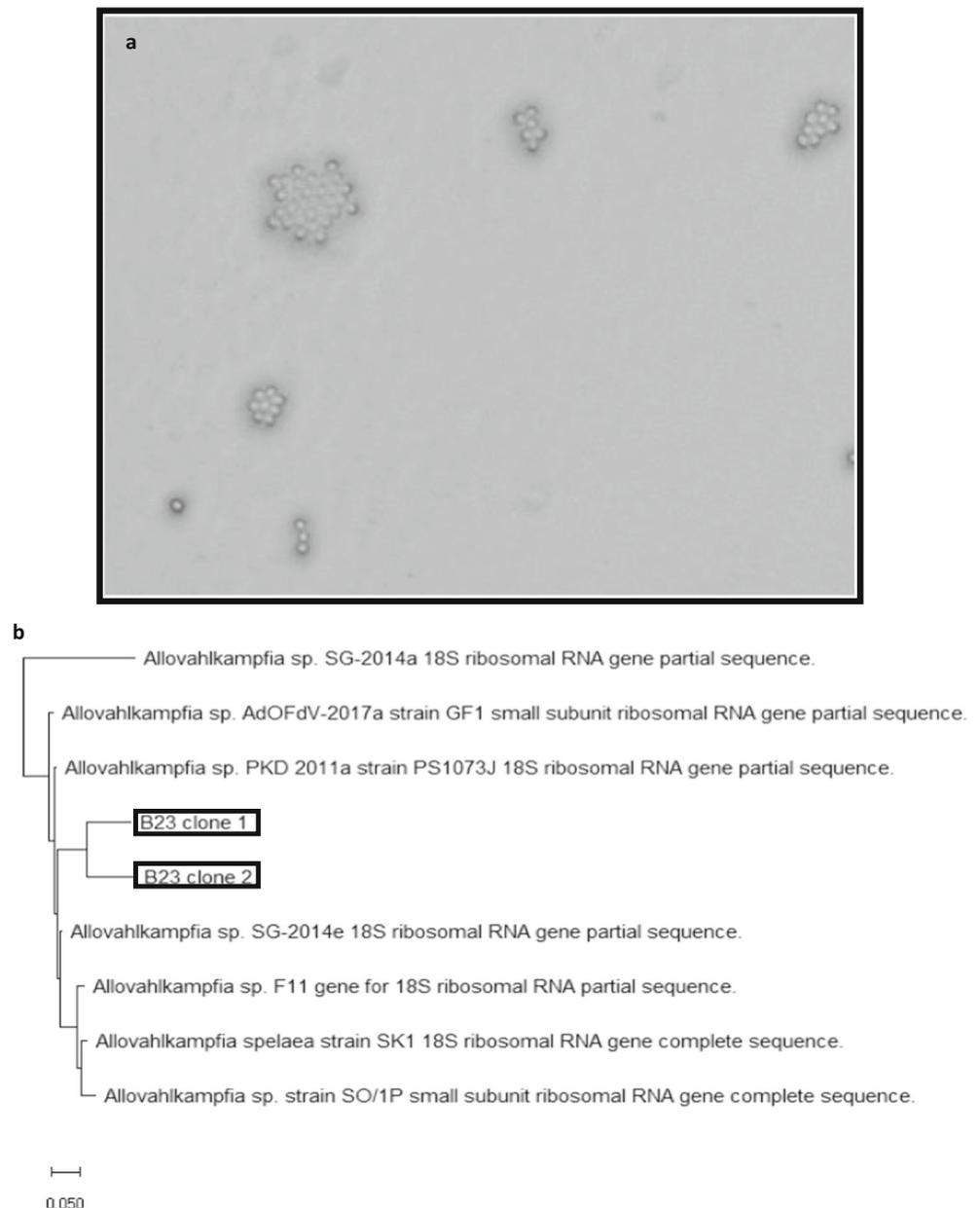


Fig. 2 **a** B23 isolate, *Allovalhkampfia* spp. cysts at $\times 20$ of magnification isolated from one of the analyzed dishcloths. **b** Relationship between the characterized *Allovalhkampfia* strains for which sequence data are known. 18S rDNA linearized neighbor-joining tree obtained by using the Kimura two-parameter distance algorithm, produced in MEGA X. The isolate obtained in the present study is identified in the tree (boxes). The type sequences were taken from GenBank



Battle et al. 2015). Some strains were lost due to bacterial overgrowth and were not able to be characterized at the molecular level.

DNA extraction

DNA from the positive samples was extracted using the Maxwell® 16 System and the Maxwell® 16 Tissue DNA purification kit sample cartridge (Promega, Madrid, Spain) following the manufacturer protocol. First of all, the NNA plates were lightly scraping using 2–4 ml of PAS and, after that, the resulting wash was centrifuged (1500 rpm for 10 min) and placed directly into the Maxwell® 16 cartridge. Amoebic

genomic DNA yield and purity were determined using the DS-11 Spectrophotometer (DeNovix®, USA).

PCR amplification

To proceed to their molecular identification, PCR analysis was performed using primers P-FLA F 5'-CGCGGTAATTCCAGCTCCAATAGC-3' and P-FLA R 5'-CAGGTTAAGGTCTCGTTGTTAAC-3' (Tsvetkova et al. 2004) and the *Acanthamoeba* specific primers JDP1 5'-GGCCAGATCGTTTACCGTGAA-3' and JDP2 5'-TCTCACAAAGCTGCTAGGGAGTCA-3' (Schroeder et al. 2001). For all PCRs, amplification reactions were performed in a

50- μ l mixture containing 80 ng DNA for FLA and 40 ng DNA for *Acanthamoeba* spp. and the PCRs were also performed in 40 and 35 cycles respectively with denaturation (95 °C, 30 s), annealing (50 °C, 30 s) and primer extension (72 °C, 30 s). After the last cycle, a primer extension was maintained for 7 min at 72 °C. Amplification products from all PCRs were analyzed by electrophoresis through a 2% agarose gel.

PCR products were sequenced using Macrogen Spain service (Avda. Sur del Aeropuerto, Madrid, Spain). Finally, the molecular identification was based on sequence analysis of 18 s rDNA genus and DF3 region as it has been previously described in comparison with the available FLA and *Acanthamoeba* DNA sequences in GenBank database (Booton et al. 2005; Niyiyati et al. 2009). *A. castellanii* Neff ATCC 30010 DNA was used as a positive control.

Phylogenetic analysis

Phylogenetic analyses were carried out using Kimura two-parameter distance algorithm, produced in MEGA X (Kumar et al. 2018). 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 the GenBank database.

Results and discussion

A total of 13 samples (42%) were positive after culturing in NNA plates and identified at the morphological level. However, and due to bacterial overgrowth, only six of the strains (46%) were used in the molecular analyses (Fig. 1a, Fig. 2a, and Table 2). After PCR performance and DNA sequence homology and phylogenetic studies, it was revealed

that *Acanthamoeba* genus was the most abundant in the PCR positive samples (5/6; 83.33%), which confirms previous studies where this genus has been described as the most common FLA genus in environmental and clinical samples (Siddiqui and Khan 2012; Lorenzo-Morales et al. 2015). Phylogenetic analysis of the obtained DF3 sequences of the *Acanthamoeba* isolates allowed the classification of them at the genotype level. The obtained results, in this case, established that four strains belonged to T4 genotype (80%) and one (1/5; 20%) to genotype T11 (Fig. 1b). Furthermore, both genotypes have been reported as causative agents of *Acanthamoeba*-related pathologies (Lorenzo-Morales et al. 2015) and even genotype T11 was reported in a keratitis case in Spain (Lorenzo-Morales et al. 2011).

One isolate was classified at the morphological and molecular level as a member of *Allovahlkampfia* genus. The isolated strain was able to form cysts in NNA plates (Fig. 2a). Moreover, homology and phylogenetic analyses confirmed that the strain belonged to this genus. However, due to the lack of many available sequences for this genus in the Genbank database, classification at the species level was not possible (Fig. 2b). Nevertheless, it is important to mention that Tolba and colleagues recently reported *Allovahlkampfia spelaea* as a causal agent of keratitis in humans (Tolba et al. 2016).

The obtained sequences have been deposited in the GenBank database under the accession numbers MH842314–MH842325 (Table 2).

Recent studies have demonstrated that FLA are ubiquitous environmental protozoa and contribute to the microbiological contamination of environmental sources (Guimaraes et al. 2016). On the other hand, FLA have shown resistance to environmental harsh conditions (Trabelsi et al. 2012), and play an important role in the control of the microbial community population (Guimaraes et al. 2016). Furthermore, this is due to its predatory behavior and microbicidal activity, although some organisms have developed resistance to the intracellular

Table 2 Sample codes of the isolated FLA strains isolated in this study. *Acanthamoeba* genotypes are shown

CODE	NNA	PCR	SPECIES	GENOTYPE	GENBANK
B5	+	–	–	–	–
B8	+	+	<i>Acanthamoeba</i> spp.	T4	MH842314/5
B11	+	+	<i>Acanthamoeba</i> spp.	T11	MH842316/7
B18	+	–	–	–	–
B19	+	–	–	–	–
B20	+	–	–	–	–
B23	+	+	<i>Allovahlkampfia</i> spp.	–	MH842318/9
B25	+	+	<i>A. polyphaga</i>	T4	MH842320/1
B26	+	+	<i>Acanthamoeba</i> spp.	T4	MH842322/3
B27	+	–	–	–	–
B30	+	+	<i>A. polyphaga</i>	T4	MH842324/5
B31	+	–	–	–	–

digestion of FLA (Greub and Raoult 2004; Torvinen et al. 2004). In the case of *Acanthamoeba* genus, its excellent role as reservoirs for amoeba-resistant microorganisms (ARM) has been reported, such as bacteria, viruses, and fungi (Guimaraes et al. 2016). Chavatte et al. (2014) revealed the dishcloths to be a potentially important source of cross-contamination with FLA and foodborne pathogens in food-related environments. In the present study, *Acanthamoeba* spp. was the most abundant isolated FLA, which is in accordance with the results obtained by Chavatte (Chavatte et al. 2014). On the other hand, other studies have also revealed *Acanthamoeba* spp. to be the most common FLA genus isolated from soil samples (Geisen et al. 2014). On account of that, we decided to evaluate the presence of FLA in dishcloths used not only in food-related zones but also used for domestic purposes (dusting, cleaning of balconies, or windows, etc). Despite the sampling size, we established that *Acanthamoeba* spp. was the most abundant FLA type among the analyzed samples. Furthermore, and to the best of our knowledge, this is the first study where FLA have been isolated from dishcloths in Spain. In addition, this is also the first isolation of *Allovalhikampfia* spp. in the Spanish territory.

Overall, our results confirm the previously obtained results from other researchers and highlight the importance of FLA as microorganisms present in food- and domestic-related environments and open a new field to further elucidate the presence of FLA in these sources and also ARM.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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