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Short Communication

A multisystemic *Acanthamoeba* infection in a dog in Tenerife, Canary Islands, Spain

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ABSTRACT

A 22-month-old male Spanish water dog was hospitalized after its physical examination revealed fever and movement difficulty. After 24 h, the dog was found to have a high fever (39.5 °C) and was treated empirically with doxycycline/ciprofloxacin. At 48 h, after submission the fever rose to 41 °C and the animal presented with a stiff neck and dehydration. Peripheral blood and cerebrospinal fluid (CSF) were sampled and trophozoites with an *Acanthamoeba*-like morphology were observed in the CSF. PCR specific for *Acanthamoeba*, *Naegleria fowleri* and *Balamuthia mandrillaris* were performed and the CSF sample found positive for *Acanthamoeba*. Lungs, kidney, liver and spleen samples were collected post mortem. All collected organ samples were positive for *Acanthamoeba* by PCR, thus confirming a multisystemic infection. Water samples taken at a suspected site of infection yielded an almost identical PCR fragment to those of the clinical samples, indicating that this was probably where the infection originated. This is the first report of a fatal case of *Acanthamoeba* disseminated infection in a dog in Spain.

1. Introduction

Opportunistic free living amoebae belonging to the genera *Acanthamoeba*, *Balamuthia* and *Naegleria* are the causative agents of encephalitis, keratitis and disseminated/multisystemic infections in humans and other animals (Siddiqui and Khan, 2012; Lorenzo-Morales et al., 2013). Infections due to these amoebae have been reported worldwide in many animals such as horses, cattle and birds. *Acanthamoeba* has been reported as causative agents of encephalitis in three dogs (Pearce et al., 1985; Bauer et al., 1993; Brofman et al., 2003), four cases of disseminated infections (Ayers et al., 1972; Dubey et al., 2005; Reed et al., 2010; Kent et al., 2011) and a case of prostatitis (Lorenzo-Morales et al., 2013). Moreover, *Balamuthia mandrillaris* has been previously reported as the causative agent of meningoencephalitis in three dogs (Foreman et al., 2004; Finnin et al., 2007; Hodge et al., 2011).

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To date *Acanthamoeba* genus is classified at the genotype level (18 different genotypes, T1–T18) based on rRNA gene sequencing (Booton et al., 2005; Nuprasert et al., 2010; Qvarnstrom et al., 2013). About 90% of *Acanthamoeba* strains isolated from infection cases have been reported as T4 genotype, although other genotypes have been reported in amoebic infection cases in humans and other animals such as T1, T3, T5, T10, T11, T15, T17 and T18 (Lorenzo-Morales et al., 2013; Qvarnstrom et al., 2013).

A case of a multisystemic infection in a dog due to *Acanthamoeba* genotype T4 is reported in this study. To the best of our knowledge, this is the first report of fatal case of *Acanthamoeba* infection in a dog in Spain and the second caused by the T4 genotype worldwide.

2. Materials and methods

2.1. Animal

A 22-month-old male Spanish water dog had suffered an episode diagnosed as a meningoencephalitis due to an unknown etiological agent 6 months before it was admitted again to the Vesal Veterinary Clinic (Tenerife, Canary Islands, Spain) with a newly developed set of symptoms including a fever and an apparent difficulty of movement.

Cerebrospinal fluid (CSF) and peripheral blood were investigated for the presence of free-living amoebae (FLA) and the day after the dog's death, kidney, lungs, spleen and liver samples were also analyzed for the presence of these pathogens.

2.2. Culture and identification of the amoebae

Amoebae were observed microscopically direct from CSF and organ samples were cultured on 2% non-nutrient agar (NNA) plates at 22 °C and 37 °C and were monitored daily for the presence of free-living amoebae as previously described (Lorenzo-Morales et al., 2005). In order to isolate FLA from a spring suspected as being a source of the infection, a liter of the water sample was filtered through a cellulose nitrate filter, 0.45 μm diameter (Millipore, Bedford, Madison) with a weak vacuum (flow rate, 1.3 ml/min). The filters were inverted on 2% NNA non-nutrient agar plates and incubated at 22 °C and 37 °C. After 3–4 days, the membranes were removed and the plates were incubated for a further 1–2 weeks. These plates were monitored for out-growth of amoebae microscopically as described above.

2.3. DNA isolation

DNA from cultures identified as being positive for FLA by microscopy was extracted by placing 1–2 ml of the amoebae 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, Madrid, Spain). DNA yield and purity were determined using the NanoDrop® 1000 spectrophotometer (Fisher Scientific, Madrid, Spain). The purified amoebic genomic DNA was then used to direct PCR.

For the identification and genotyping of *Acanthamoeba* isolates, the diagnostic fragment 3 region (DF3) of *Acanthamoeba* 18S rDNA gene was amplified as previously described using the JDP1/JDP2 primer pair (Booton et al., 2005; Niyyati et al., 2009). The primers used are JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′). This PCR amplifies a typically 495 bp fragment of the 18S rDNA gene that allows genotype identification after sequencing.

The resulting 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 Services (Servicio de Secuenciación SEGAI, University of La Laguna). Sequences were aligned using Mega 5.0 software program (Tamura et al., 2011). Genotype identification was based on sequence analysis of DF3 region as previously described by comparison to the available *Acanthamoeba* DNA sequences in Genbank database (Booton et al., 2005; Niyyati et al., 2009). *Acanthamoeba castellanii* Neff ATCC 30010 DNA was used as a positive control in the PCR reactions.

The presence of suspected microorganisms was tested by a number of culture methods including Blood Agar plates (anaerobic and aerobically incubated), McConkey Agar, Sabouraud's Agar and brain-heart broths. PCRs specific for Canine distemper virus, *Acanthamoeba*, *Naegleria fowleri* and *Balamuthia mandrillaris* were performed and the in the case of *Naegleria* spp. PCR, the 18S rDNA gene (Dykova et al., 2001) and the ITS typing region (De Jonckheere and Brown, 2005) were amplified as previously reported. For the identification of *Balamuthia mandrillaris*, the 16S rDNA gene was performed as previously described (Niyyati et al., 2009). Immunoglobulin levels of the infected dog were measured by the standard radial immunodiffusion method (Mancini et al., 1965).

Phylogenetic analyses for the obtained sequences were carried out using maximum parsimony, minimum evolution and maximum likelihood optimality criteria, implemented in Mega 5.0 (Tamura et al., 2011). 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 isolate are deposited in the Genbank database under the accession numbers: KJ439562, KJ439566.

3. Results and discussion

Most of the previously reported cases of amoebal infections in dogs were manifesting as encephalitis or multisystemic disseminated infections (Ayers et al., 1972; Bauer et al., 1993; Brofman et al., 2003; Dubey et al., 2005; Kent et al., 2011). Although the symptoms of multisystemic infection of *Acanthamoeba* in the dog are variable, typical cases involve the loss of appetite, fever, discharges from the nose and eyes, and neurological signs such as neck and limb stiffness. It is apparent from these previous reports that

Table 1
Reported genotypes of *Acanthamoeba* in dogs.

Age/Dog type		Genbank	Reference
One-year-old Labrador	T1	N/A	Dubey et al. (2005)
10 month old boxer	T1	GQ924681 GQ924682	Kent et al. (2011)
10 year old mongrel	T4	JN555599	Lorenzo-Morales et al. (2013)
22 month old Spanish water dog	T4	KJ439562 KJ439566	This study

Table 2a
Blood counts.

	Counts	Reference range
White blood cells	24.8×10^3 cells/μL	$6.0–17.0 \times 10^3$ cells/μL
Lymphocytes	10%	12–30%
Staff cells	14%	0–3.0%
Eosinophils	0.0%	2–10%
Sodium	146 meq/L	128–150 meq/L
Potassium	5 meq/L	3.50–5.0 meq/L
Alkaline phosphatase	258 U/L	32–185 U/L
Transaminase GOT	82 U/L	19–70 U/L
Total IgA	84.70 mg/dL	40–160 mg/dL
Total IgG	456 mg/dL	700–2000 mg/dL
Total IgM	120 mg/dL	100–200 mg/dL

younger dogs are more likely to succumb to *Acanthamoeba* infection as are immunosuppressed dogs.

The subject of the present study was hospitalized after its physical examination which revealed fever and movement difficulty that the dog was showing for 5 days prior to its admission. After 24 h, the dog presented with a high fever (39.5 °C) and was treated empirically with doxycycline (100 mg each 12 h) and ciprofloxacin (250 mg each 8 h). At 48 h after admission, the animal's fever reached 41 °C and it showed a stiff neck and dehydration. At this stage, peripheral blood and cerebrospinal fluid were extracted and trophozoites were observed in the CSF and cultured. DNA was extracted directly from the cultures and DF3 fragment PCR (*Acanthamoeba*) was carried out in order to verify the microscopy observations. PCR was also positive for these cultures and after purification. The obtained sequences (direct CSF and culture of CSF) revealed that this isolate was *Acanthamoeba* genotype T4.

Complete blood count revealed an increased number of total white blood cells (WBC) count and the CSF analysis also showed a large increase in the WBC count (Tables 2a and 2b). The observed alterations in the WBC count, the hepatic enzymes and the IgG levels could all have played an important role in the disseminated infection.

Table 2b
CSF analysis.

	Counts	Reference range
White blood cells	5800 cells/μL	0–6.0 cells/μL
Neutrophils	40%	
Monocytes	49%	
Lymphocytes	11%	
Protein level	145 mg/dL	0.24–0.3 mg/dL
Glucose	91 mg/dL	40–70 mg/dL

All collected organ samples were also positive for *Acanthamoeba*, thus confirming a multisystemic infection in the animal. PCR were positive in all tested organs and, the obtained sequences were identical to the CSF ones and confirmed the classification of this isolate into *Acanthamoeba* genotype T4. Interestingly, lung samples presented a high number of trophozoites and cysts of *Acanthamoeba* even directly upon observation of the organ sample under the microscope (Fig. 1A).

The dog's owner reported that the dog actively played, and drank water around a natural spring in a recreational area in the area of Mesa Mota (N 28° 30′ 35.151″ W 16° 19′ 37.392″), San Cristóbal de La Laguna, Tenerife, Spain. Therefore, samples were collected from this spring and were checked for the presence of *Acanthamoeba* and other free-living amoebae. Interestingly, *Acanthamoeba* was isolated from the samples and PCR followed by sequencing of the DF3 region revealed that the strain also belonged to genotype T4. Homology analysis revealed that the obtained sequences were 99.9% homologous between the suspect spring water isolate and the isolates from the dog infection (Fig. 1C). This level of similarity makes it very likely that t the strain that infected the animal came from this natural spring (Fig. 1B).

Acanthamoeba genotype T4 is the genotype most often associated with human infection (Booton et al., 2005) but it is also the most commonly isolated genotype from the environment. However a statistical analysis shows that the frequency of T4 association with human disease is not explained by its frequency in the environment alone (Maciver et al., 2013) and so in humans at least T4 is specifically pathogenic. Previous work from our group has shown than potentially pathogenic *Acanthamoeba* strains are present in a variety of sources on the island of Tenerife (Lorenzo-Morales et al., 2005, 2007) and Gran Canaria (Reyes-Batlle et al., 2014). The conclusion of these studies is that as elsewhere T4 is the dominant genotype in the general environment of the Canary Islands. To date too few infections with *Acanthamoeba* in dogs have been genotyped (Table 1) to determine if T4 is also the most frequently infective genotype as it is in humans and for AK in cats (Ithoi et al., 2013). Interestingly, two previous cases of *Acanthamoeba* infection in dogs were found to be T1 (Dubey et al., 2005; Kent et al., 2011), a genotype that has been found to infect humans, but at very low frequency. We are aware of only one other study in which the genotype of a dog infection was found to belong to the T4 group and this was a recent study by our laboratory on a dog with prostatitus also from Tenerife (Lorenzo-Morales et al., 2013). The T4 strains in both Tenerife cases are only 97% identical (Fig. 1C) indicating that they are not the same strain of *Acanthamoeba*.

A recent study of *Acanthamoeba* isolated from skin lesions and nasal mucosa of dogs in Porto Alegre, Brazil, 5 out of 13 isolates were T4 with 4x T5s, 3x T3s and one T16 (Carlesso et al., 2014). Although there was no evidence for these *Acanthamoeba* being the cause of the lesions it is possible that pathogenic amoebae gain entry to the dogs through these sites and possibly the nasal mucosa. To the

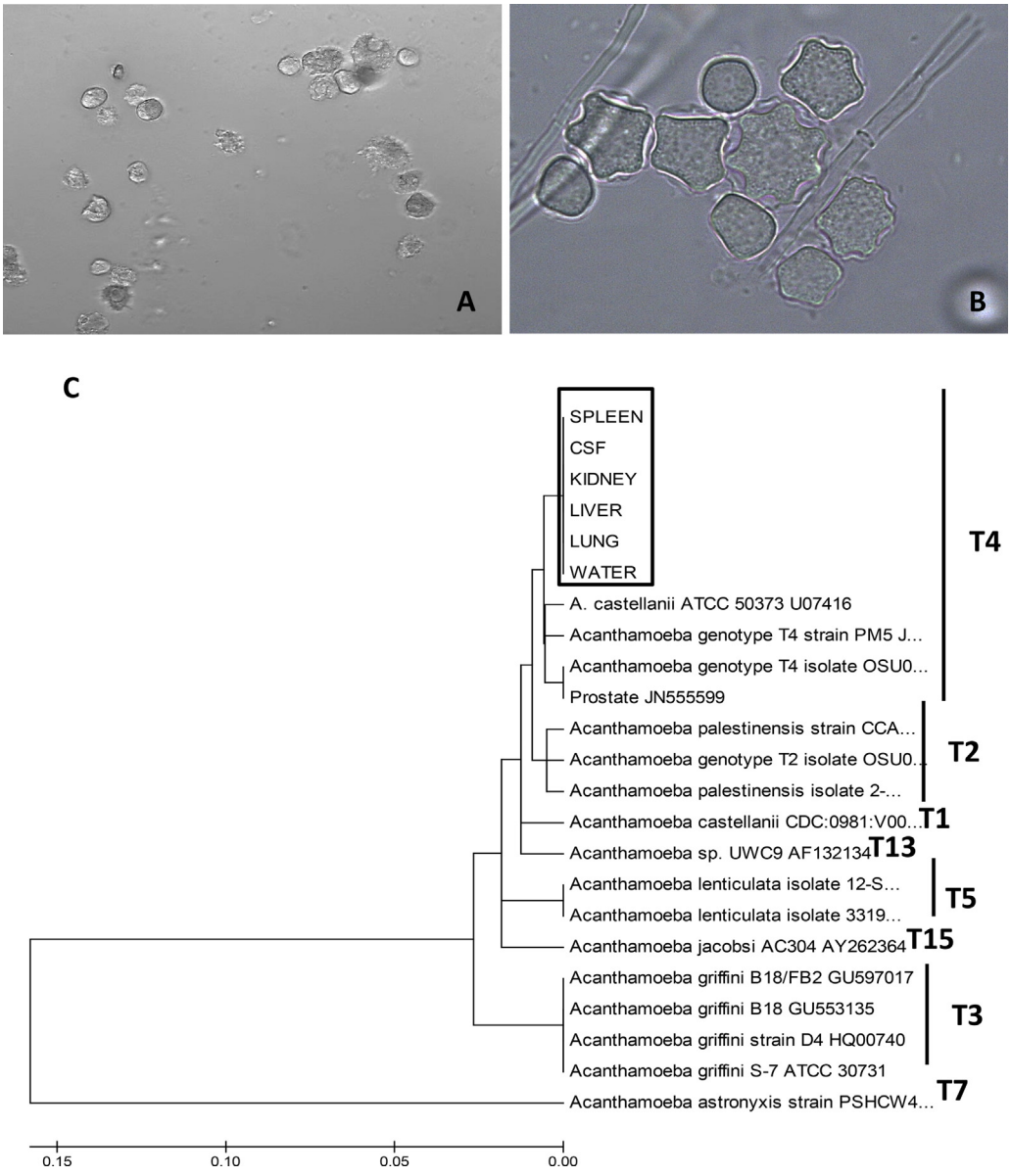

Fig. 1. (A) *Acanthamoeba* trophozoites and cysts (20×) in the lung biopsy. (B) Presence of *Acanthamoeba* cysts (100×) in the water sample. (C) Phylogenetical analysis of the DF3 sequence of the isolated *Acanthamoeba* strains from CSF and organs samples (spleen, kidney, liver, and lung form the infected dog as well as the possible infection source (water) belonged to genotype T4 and were 99.9% identical. These sequences were 97% identical to the strain previously isolated from a dog in Tenerife (Lorenzo-Morales et al., 2013) (JN555599).

best of our knowledge, this is the first report of fatal case of *Acanthamoeba* disseminated infection in a dog in Spain and the second caused by the T4 genotype.

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