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ORIGINAL PAPER

Molecular characterization of *Acanthamoeba* strains isolated from domestic dogs in Tenerife, Canary Islands, Spain

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Abstract The present study describes two cases of *Acanthamoeba* infections (keratitis and ascites/peritonitis) in small breed domestic dogs in Tenerife, Canary Islands, Spain. In both cases, amoebic trophozoites were observed under the inverted microscope and isolated from the infected tissues and/or fluids, without detecting the presence of other viral, fungal or bacterial pathogens. Amoebae were isolated using 2 % non-nutrient agar plates and axenified for further biochemical and molecular analyses. Osmotolerance and thermotolerance assays revealed that both isolates were able to grow up to 37 °C and 1 M of mannitol and were thus considered as potentially pathogenic. Moreover, the strains were classified as highly cytotoxic as they cause more than 75 % of toxicity when incubated with two eukaryotic cell lines. In order to classify the strains at the molecular level, the diagnostic fragment 3 (DF3) region of the 18S rDNA of *Acanthamoeba* was amplified and sequenced, revealing that both isolates belonged to genotype T4. In both cases, owners of the animals did not allow any further studies or follow-up and therefore the current status of these animals is unknown. Furthermore, the isolation of these pathogenic amoebae should raise awareness with the veterinary community locally and worldwide.

Keywords *Acanthamoeba* · Dog · Infection · Keratitis · Ascites · Canary Islands · Spain

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Introduction

Free-living amoebae belonging to the genus *Acanthamoeba* and the species *Naegleria fowleri* and *Balamuthia mandrillaris* are causative agents of lethal encephalitis and multisystemic infections as well as keratitis (in the case of *Acanthamoeba* genus) in humans and other animals (Siddiqui and Khan 2012; Lorenzo-Morales et al. 2013).

There are reports worldwide of these opportunistic pathogens as causative agents of infections in various members of the animal kingdom such as cattle, birds and horses. Focusing on *Acanthamoeba* genus and infections in domestic animals, these amoebae have been reported as causative agents of encephalitis in three dogs (Pearce et al. 1985; Bauer et al. 1993; Brofman et al. 2003), five cases of disseminated infections (Ayers et al. 1972; Dubey et al. 2005; Reed et al. 2010; Kent et al. 2011; Valladares et al. 2014) and a case of prostatitis (Lorenzo-Morales et al. 2013). Furthermore, *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). No natural cases of *Naegleria fowleri* infections in domestic animals have been described so far.

To date, *Acanthamoeba* genus is classified at the genotype level (19 different genotypes, T1–T19) based on rRNA gene sequencing (Booton et al. 2005; Nuprasert et al. 2010; Qvarnstrom et al. 2013; Magnet et al. 2014). Strains of *Acanthamoeba* belonging to genotype T4 have been isolated in 90 % of the reported clinical cases worldwide, although genotypes such as T1, T3, T5, T10, T11, T15, T17 and T18 are also related to amoebic infection cases in humans and other animals (Lorenzo-Morales et al. 2013; Qvarnstrom et al. 2013).

In this study, *Acanthamoeba* strains belonging to genotype T4 were isolated from a case of canine keratitis and ascites/peritonitis. To the best of our knowledge, this is the first report on the isolation of potentially pathogenic *Acanthamoeba* strains in dogs suffering the conditions mentioned above in Spain and worldwide.

Materials and methods

Animal samples

Corneal swabs and tears were collected from a dog suffering from keratitis. In the case of the dog suffering with ascites, the fluid of the peritoneal cavity was collected and kept in sterile tubes until culture. It is important to mention that no bacterial, viral, fungal or yeast agents were identified in these samples and therefore were submitted to our laboratory to check for the presence of free-living amoebae.

Culture and identification of the amoebae

Amoebae were observed under the microscope directly from peritoneal fluid, and corneal swab samples were cultured on 2 % non-nutrient agar (NNA) plates at 22 and 37 °C and were monitored daily for the presence of free-living amoebae as previously described (Lorenzo-Morales et al. 2006) with an overlayer of *Escherichia coli* suspension that had been heat inactivated (2 h at 60 °C). The isolates were cultured axenically in 75-cm^2 culture tissue flask without shaking, at 28 °C, in PYG medium [0.75 % (wt/vol) proteose peptone, 0.75 % (wt/vol) yeast extract and 1.5 % glucose] supplemented with 20 µg/ml of gentamicin.

DNA isolation

DNA from cultures identified as positive for amoebae by microscopy was extracted by placing 1–2 ml of amoeba 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).

18S rDNA gene amplifications for *Acanthamoeba* (DF3 region) were performed as previously described (Booton et al. 2005; Niyyati et al. 2009). 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.

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 isolates are deposited in the GenBank database under the accession numbers: KP728281-KP728282.

Tolerance assays

Osmotolerance of the isolated amoebae was assayed by incubating the strains onto 2 % non-nutrient agar plates containing mannitol 0.5, 1.0 and 1.5 and seeded with heat-killed *E. coli*. Approximately 10^3 trophozoites were inoculated onto the centre of non-nutrient agar plates. Plates were observed for amoebae growth with an inverted microscope after 24, 48 and 72 h. To investigate thermotolerance, approximately 10^3 trophozoites were inoculated in the centre of non-nutrient agar plates seeded with heat-killed *E. coli*. The plates were incubated at 28, 37 and 42 °C and monitored using an inverted microscope for amoebae growth after 24, 48 and 72 h. Approximately 10^3 trophozoites were inoculated in the centre of new non-nutrient agar plates seeded with heat-killed *E. coli* incubated at room temperature, and monitored for growth after 24, 48 and 72 h. This plate was used as a control. The procedure was repeated using previously established thermo- and osmotolerant *Acanthamoeba* strains as positive controls (Martín-Navarro et al. 2008).

Cytotoxicity assays

The two cell lines that were used in this study, J774A.1 murine macrophages (ATCC # TIB-67) and HeLa cells (ATCC # CCL-2), were maintained as monolayer cultures at 37 °C in 5 % CO_2 in T-75 culture flasks (Corning, Madrid, Spain). Cells were routinely cultured in Dulbecco's modified Eagle medium without phenol red (DMEM, Sigma, Tres Cantos, Madrid, Spain) supplemented with 10 % foetal calf serum, 2 mM glutamine and 20 µg/ml gentamicin (Biochrom AG, Cultek, Granollers, Barcelona, Spain) and subcultured two times per week.

Cytotoxicity assays were performed using a cytotoxicity detection kit based on the release of lactate dehydrogenase enzyme (LDH) (Roche Diagnostics, Sant Cugat del Vallès, Barcelona, Spain) as previously described (Lorenzo-Morales et al. 2005; Martín-Navarro et al. 2010). Briefly, the two cell lines were grown to monolayers in 6-well plates. Both isolated *Acanthamoeba* strains (10^5 amoebae/well) were incubated with the cell monolayers in foetal calf serum-free DMEM at 37 °C in 5 % CO_2. Cell monolayers were observed periodically for cytopathic effects for up to 24 h. At the end of this incubation period, cytopathic effects were assessed visually after haematoxylin staining. In addition, supernatants were collected and cytotoxicity was determined by measuring LDH release following manufacturer's instructions. Control values were obtained from each cell line incubated alone in foetal calf serum-free DMEM without phenol red. Total LDH release was determined from each cell line treated with 2 % Triton X-100 (Sigma, Tres Cantos, Madrid, Spain).

Results and discussion

In both cases reported in this study, no other pathogen was isolated from the eye or the peritoneal fluid of the animals. Therefore and due to recent descriptions of amoebic infection cases in the Canary Islands, samples were submitted to our laboratory to check for the presence of potentially pathogenic free-living amoebae.

Upon observation of corneal swab and peritoneal fluid microscopically, the presence of amoebic trophozoites was revealed. After that, drops of peritoneal fluid and part of the corneal swab were cultured in 2 % NNA plates for the isolation of the amoebae. After this process, DNA was extracted directly from the samples and also from the amoebic cultures and DF3 fragment PCR (*Acanthamoeba*) was carried out in order to verify the microscopy observations. PCRs were also positive for these cultures and after purification (Fig. 1). Furthermore, the obtained sequences (direct fluid/swab and culture of fluid/swab) allowed the classification of both isolates into *Acanthamoeba* genotype T4. Both strains were able to grow at temperatures up to 37 °C and 1 M of mannitol, induced high cytotoxicity levels when tested against the eukaryotic cell lines and were thus considered as pathogenic (Table 1).

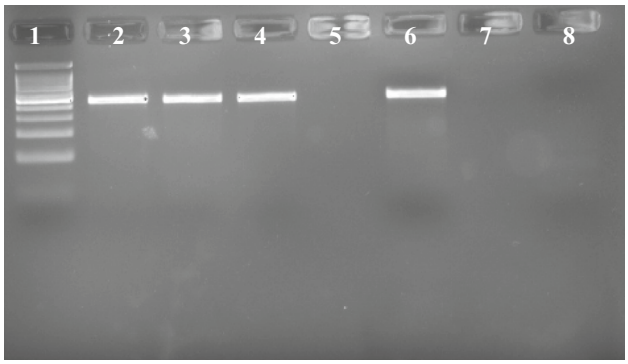

Fig. 1 Diagnostic fragment 3 PCR of the tested samples. *Lane 1* 100 bp DNA ladder; *lane 2* corneal swab; *lane 3* peritoneal fluid; *lane 4* peritoneal fluid culture in NNA plates; *lane 6* positive control *Acanthamoeba castellanii* Neff ATCC 30100 DNA; *Lane 7* negative control. *Lanes 5* and *8* are empty

Table 1 (A) Characteristics of the isolated strains in this study. (B) Cytotoxicity levels induced by the isolated strains when incubated with murine macrophages and HeLa cells

Isolate	Source	Pathogenic potential		Genotype
		Temperature tolerance 37 °C	Osmotolerance 1 M mannitol	
(A)				
TFD1	Corneal swab	+++	+	T4
TFD2	Peritoneal fluid	+++	++	T4
Isolate		Eukaryotic cell line		
Toxicity		J774.1		HeLa
(B)				
TFD1		78 ± 3.75		79 ± 2.01
TFD2		81 ± 4.03		91 ± 3.52

Most of the previously reported cases of infections in dogs (Ayers et al. 1972; Bauer et al. 1993; Brofman et al. 2003; Dubey et al. 2005; Kent et al. 2011; Valladares et al. 2014) were manifesting as encephalitis or multisystemic disseminated infections. 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. Younger dogs are more likely to succumb to *Acanthamoeba* infection as are immunosuppressed dogs.

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 in the Canary Islands (Lorenzo-Morales et al. 2005; 2007; Reyes-Batlle et al. 2014). The conclusion of these studies is that as elsewhere T4 is the dominant genotype. To date, too few infections with *Acanthamoeba* in dogs have been genotyped to determine whether 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 three cases in which the genotype of a dog infection was found to be T4 and two of them were recent reports by our laboratory in dogs (one case of prostatitis and a case of multisystemic infection) from Tenerife (Lorenzo-Morales et al. 2014; Valladares et al. 2014). The T4 strains in both Tenerife cases are only 97 % identical indicating that they are not the same strain of *Acanthamoeba* (Valladares et al. 2014).

In a recent study of *Acanthamoeba* isolated from skin lesions and nasal mucosa of dogs in Porto Alegre, Brazil, five out of thirteen isolates were T4 with four T5s, three 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. In the reported case, the slight immunosuppression stage that was observed seems to have played an important role in the progress of the infection. In the reported cases in our study, the cause of infections was not established since the owners did not allow further studies in the animals and rejected to give further information about the status and the environments that both animals were experiencing. To the best of our knowledge, this is the first report on the isolation of potentially pathogenic *Acanthamoeba* strains in dogs suffering the conditions mentioned above in Spain and worldwide.

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