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CASE REPORT

Regional spread of contact lens-related *Acanthamoeba* keratitis in Italy

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SUMMARY

Acanthamoeba ocular infections, known as *Acanthamoeba* keratitis, are an emerging problem among contact lens wearers. Infections mediated by *Acanthamoeba* are uncommon, but they can be underestimated due to poor awareness and delayed diagnosis. The routine use of rapid and cost-effective molecular methods like Real Time PCR for the diagnosis of this important pathogen could improve diagnosis and therapy outcome. This report describes the detection by Real Time PCR assay of six T4 and one T3 *Acanthamoeba* infections, as the first reported cases in Tuscany, Italy.

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INTRODUCTION

Acanthamoebae are ubiquitous free-living protozoa that can cause severe corneal infections in humans, known as *Acanthamoeba* keratitis (AK) (Cerulli *et al*., 1998; Hamburg *et al*., 2009). Despite being uncommon, the concomitant damage to the corneal epithelium barrier (trauma or contact lens-related) and exposure to *Acanthamoeba* significantly increases the risk of infection also in immunocompetent individuals (Alkharashi *et al.,* 2015; Feher *et al.,* 2009; Marciano-Cabral, 2009). In fact, the incidence of *Acanthamoeba* infections has risen in both low and high income countries with the increased use of contact lenses (Joslin *et al.,* 2007; Pacella *et al.*, 2012; Pacella *et al.*, 2013). Despite this general increase, the prevalence of AK may significantly differ in different geographic areas. In particular, in United Kingdom the incidence of AK is significantly higher than in the United States (17.53-19.50 *vs* 1.65-2.01 cases per million contact lens wearers, respectively) (Cheung *et al*., 2016).

Detection of this important pathogen is achievable by microscopy, cultural and molecular methods, whereby the use of molecular techniques has notably increased diagnostic sensitivity (Lorenzo Morales *et al.*, 2015). However, due to the low incidence and poor awareness of *Acanthamoeba* infections, the diagnosis is often delayed and based on exclusion criteria when the corneal damage is already severe (Gatti *et al.,* 2010). Indeed, the clinical symptoms often resemble fungal or herpes infections, and the differential diagnosis is frequently postponed with a consequent inappropriate empirical therapy (Maycock and Jayswal, 2016). Moreover, up to 23% of *Acanthamoeba* infections are polymicrobial, and co-infection further increases the possibilities of an erroneous identification of AK (Maycock and Jayswal, 2016).

In Italy, the first case of *Acanthamoeba* corneal infection was described in the early 1990s (Manso *et al.,* 1993). Thereafter, other AK infections have been reported from various Italian regions (Gatti *et al.,* 2010; Di Cave *et al.*, 2009; Di Cave *et al.*, 2014), but no cases have been reported so far from Tuscany.

This report describes the first seven cases of *Acanthamoeba* corneal infections from Tuscany, Italy, and a novel Real Time PCR (RT-PCR) for rapid detection of this pathogen.

CASE REPORTS

From December 2013 to August 2015, 13 corneal scrapings and/or contact lens liquid samples were collected at the Eye Clinic of the Department of Surgery and Translational Medicine of Florence Careggi University Hospital (Florence, Italy) from patients with clinically suspect AK. The suspicion was based on the presence of a corneal ulcer in a contact lens user reporting poor hygiene habits, and the evidence of suspicious findings at confocal microscopy. Samples were analyzed at the Microbiology and Virology Unit of the Hospital by a combination of endpoint PCR (Schroeder *et al.,* 2001) and cultural methods, as previously described (Di Cave *et al.*, 2009; Di Cave *et al.*, 2014), and by a novel RT-PCR assay as described below.

For molecular analysis, DNA was extracted with the NucliSens® EasyMag® instrument (bioMérieux, Marcy l'Étoile,

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France). *Acanthamoeba* identification and genotyping was carried out by end-point PCR using the genus specific primers JDP1 and JDP2 to amplify a region of approximately 500 bp (ASA.S1) including the Diagnostic Fragment 3 (DF3) from the 18S-rRNA gene (Schroeder *et al.,* 2001), and subsequent sequence analysis. All procedures were performed as previously described (Di Cave *et al.,* 2009). Purified amplicons were sequenced on both strands, aligned with consensus sequences by using ClustalW2, and edited with FinchTV 1.4 (Geospiza, Inc, Seattle, WA). The neighbor-joining distance tree was constructed by comparison of the obtained sequences with selected GenBank sequences of *Acanthamoeba* reference strains of all the available genotypes using MEGA version 6 (bootstrap test 1000 replicates) (Tamura *et al.*, 2013). GenBank accession numbers of the retrieved sequences are available in *Figure 1*. A representative sequence of *Balamuthia mandrillaris* (accession number KF874819) was used as outgroup for the analysis. The evolutionary distances were computed using the Tamura 3-parameter method. The best model of tree construction was selected using jModelTest by the Akaike Information Criterion (AIC) (Posada, 2009).

The same primers used for genotyping (Schroeder *et al.*, 2001) were adapted for RT-PCR, which was run on a Rotor Gene Q instrument (QIAGEN, Venlo, Netherlands), using a Gotaq qPCRMaster Mix (Promega, USA) containing BRYT Green® dye, with 5 min at 95°C, followed by 45 cycles of 30s at 95°C, 20s at 60°C and 30s at 72°C, and final melting curve analysis (ramp from 70°C to 95°C, rising 0.5°C per step, waiting 5s per step). The melting curve temperature of positive samples was 84.3±0.2°C. Cultural analysis was carried out by plating samples on non-nutrient agar as described previously (Di Cave *et al.*, 2009).

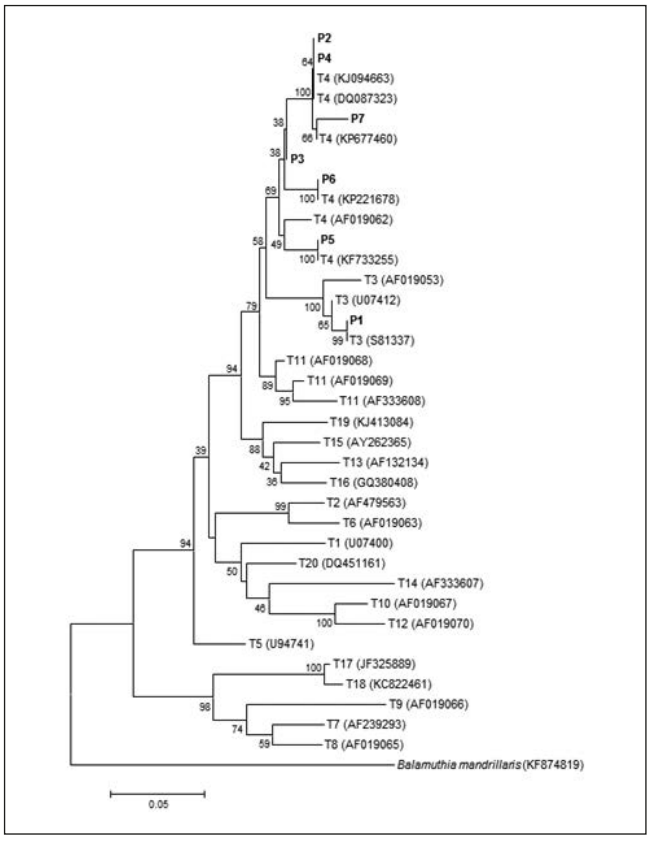

Figure 1 - *Neighbor-joining tree showing the genetic relationships among seven Acanthamoeba isolates examined in this study, based on ASA.S1 DNA sequences. GenBank accession numbers of reference strains are in parentheses.*

Table 1 - *Clinical and parasitological features of the patients with Acanthamoeba infection.*

Patient	*Age*	*Sex*	*City of origin*	*Infected eye*	*Clinical presentation*	*Severity and complications*	*Date of diagnosis*	*Therapy*	*Time of therapy*	*Material*	*Genotype*
1	40	F	Carrara (MS)[a]	right	wide corneal ulcer	Severe	Dec, 2013	PHMB[b] 0.02% + chlorhexidine 0.02%	6 months	corneal scraping	T3
2	49	M	Forte dei Marmi (LU)	right	wide corneal abrasion	Severe	Nov, 2014	PHMB + hexamidine 0.1%	-[c]	corneal scraping	T4
3	41	M	Carrara (MS)	left	central corneal ulcer	Moderate	Nov, 2014	PHMB 0.02%	7 months	corneal scraping	T4
4	22	M	Massa (MS)	left	wide corneal ulcer	Severe, corneal thinning	Feb, 2015	PHMB 0.02%+ hexamidine 0.1%	7 months	corneal scraping	T4
5	41	F	Arezzo	left	central corneal abscess with hypopyon	Severe	Jan, 2015	PHMB 0.02% + hexamidine 0.1%	5 months	corneal scraping	T4
6	29	M	Reggello (FI)	right	peripheral corneal ulcer temporal quadrant	Severe	Feb , 2015	PHMB 0.02%+ hexamidine 0.1%	-[c]	corneal scraping	T4
7	69	F	Florence	right	wide corneal central deepithelialization with hypopyon	Severe	Aug, 2015	Chlorhexidine 0.02% + PHMB 0.02% + hexamidine 0.1%	>8 months	corneal scraping[d]	T4

[a]MS: Massa Carrara; LU:Lucca; FI:Florence;
[b]polyhexamethylene biguanide;
[c]unknown. Lost to follow-up;
[d]Lens liquid was also investigated and tested positive for *Acanthamoeba* with both cultural and molecular methods.

Altogether, seven cases of *Acanthamoeba* keratitis were diagnosed by molecular analysis, among 13 suspected cases. The RT-PCR yielded consistent results with conventional end-point PCR and allowed the rapid detection and confirmation of *Acanthamoeba* infection in only 120 minutes. Six samples were also confirmed by cultural methods (*Table 1*), but in these cases one week was necessary for diagnosis, underscoring the advantage of molecular methods for rapid diagnosis. All samples negative by RT-PCR were also negative by cultural analysis.

Genotyping by ASA.S1 sequence analysis revealed that six of the seven cases belonged to genotype T4 (*Figure 1*), which was previously found to be the most common genotype from *Acanthamoeba* keratitis in Italy and worldwide (Di Cave *et al.*, 2009; Maciver *et al.*, 2013). One case was assigned to genotype T3, reported to be less common in Italy (Di Cave *et al*., 2014).

To the best of our knowledge this is the first report of *Acanthamoeba* keratitis in Tuscany. Patients were resident in different places (*Table 1*). Interestingly, four patients were from the same geographical area, located in northwestern Tuscany. Further studies will be necessary to assess the possible causes of clustering of *Acanthamoeba* infections in this area. All patients were contact lens wearers, and presented with moderate to severe corneal conditions. The mean age was 41 years, the infection was in all cases monolateral, and symptoms ranged from severe ocular pain with eye-swelling, redness and photophobia, to just mild pain. At slit-lamp examination, all patients presented a corneal ulcer in various stages of development (from small to extensive). Two of them showed hypopyon. Diagnosis was achieved from two weeks up to two months from the appearance of the first symptoms, due to the lack of specific symptoms and subsequent delay in the request for *Acanthamoeba* testing.

All patients were treated with solutions of polyhexamethylene biguanide (PHMB) 0.02%, but for some of them additional treatments (hexamidine 0.1% and/or chlorexidine 0.02%) were also necessary. A therapy of at least six months was prescribed in all cases and, for the last case, more than eight months of therapy were required (*Table 1*). In particular, patient number 7 still tested positive for *Acanthamoeba* (with both molecular and cultural methods) after eight months of continuous therapy. In these cases a combined therapy with higher concentrations of PHMB and chlorhexidine should be evaluated.

DISCUSSION

Ocular infections caused by *Acanthamoeba* are still underestimated due to diagnostic difficulties. The use of a rapid and cost-effective method of Real Time PCR for *Acanthamoeba* detection could avoid delayed diagnosis and improve therapy outcome. Different protocols of Real Time PCR for *Acanthamoeba* have been described (Qvarnstrom *et al*., 2006; Rivière *et al*., 2006; Karsenti *et al*., 2017), and this approach proved to be up to 50-fold more sensitive than conventional cyst counting (Ikeda *et al*., 2012). Interestingly, a significant number of free-living amoebae keratitis cases caused by *Hartmannella* and *Vahlkampfia* has recently been described in Sardinia (Pinna *et al*., 2017). This suggests that *Acanthamoeba* might not be the only causative agent of free-living amoebae keratitis in our territory and further emphasizes the need to implement molecular techniques for the detection of free-living amoebae keratitis.

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References

Alkharashi M., Lindsley K., Law HA., Sikder S. (2015). Medical interventions for *Acanthamoeba* keratitis. *Cochrane Database Syst Rev.* **2**, CD010792.

Cerulli L., Mancino R., Palma S., Tomei M. (1998). Cheratiti da *Acanthamoeba*. In: Scuderi G, editor. La Cornea. Masson. **14**.

Cheung N., Nagra P., Hammersmith K. (2016). Emerging trends in contact lens-related infections. *Curr Opin Ophthalmol*. **27**, 327-332.

Di Cave D., Monno R., Bottalico P., Guerriero S., D'Amelio S., et al. (2009). *Acanthamoeba* T4 and T15 genotypes associated with keratitis infections in Italy. *Eur J Clin Microbiol Infect Dis*. **28**, 607-612.

Di Cave D., D'Alfonso R., Dussey Comlavi K.A., D'Orazi C., Monno R., et al. (2014). Genotypic heterogeneity based on 18S-rRNA gene sequences among *Acanthamoeba* isolates from clinical samples in Italy. *Exp Parasitol.* **12**, 3417-3427.

Feher J., Kovacs I., Pacella E., Keresz S., Spagnardi N., et al. (2009). Pigment epithelium-derived factor (PEDF) attenuated capsaicin-induced neurotrophic keratouveitis. *Invest Ophthalmol Vis Sci*. **50**, 5173-5180.

Gatti S., Rama P., Matuska S., Berrilli F., Cavallero A., et al. (2010). Isolation and genotyping of *Acanthamoeba* strains from corneal infections in Italy. *J Med Microbiol.* **59**, 1324-1330.

Hamburg A., De Jonckheere J.F. (1980). Amoebic keratitis. *Ophthalmologica*. **181**, 74-80.

Ikeda Y., Miyazaki D., Yakura K., Kawaguchi A., Ishikura R., et al. (2012). Assessment of real-time polymerase chain reaction detection of *Acanthamoeba* and prognosis determinants of *Acanthamoeba* keratitis. *Ophthalmology*. **119**, 1111-1119.

Joslin C.E., Tu E.Y., Shoff M.E., Booton G.C., Fuerst P.A., et al. (2007). The association of contact lens solution use and *Acanthamoeba* keratitis, *Am. J Ophthalmol.* **144**, 169-180.

Karsenti N., Lau .R, Purssell A., Chong-Kit A., Cunanan M., et al. (2017). Development and validation of a real-time PCR assay for the detection of clinical acanthamoebae. *BMC Res Notes*. **10**, 355.

Lorenzo-Morales J., Khan N.A., Walochnik J. (2015). An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite.* **22**, 1-20.

Maciver S.K., Asif M., Simmen M.W., Lorenzo-Morales J. (2013). A systematic analysis of *Acanthamoeba* genotype frequency correlated with source and pathogenicity: T4 is confirmed as a pathogen-rich genotype. *Eur J Protistol.* **49**, 217-221.

Maycock NJ, Jayaswal R. (2016). Update on *Acanthamoeba* Keratitis: Diagnosis, Treatment, and Outcomes. *Cornea.* **35**, 713-20.

Manso E., Biavasco F., Lupidi R., Giovannini A., Frongia G., et al. (1993). Primo isolamento in Italia di un ceppo di *Acanthamoeba* da un paziente affetto da cheratite bilaterale, *Microbiol Med.* **8**, 100-104.

Marciano-Cabral F. (2009). Free-living amoebae as agents of human infection. *J Infect Dis.* **199**, 1104-1106 .

Pacella E., Pacella F., Scavella V., Impallara D., Turchetti P., et al. (2012). The role of contact lenses and ocular TRAUMA in determining *Acanthamoeba* Keratitis: a case-control study in Italy. *Italian J Public Health*. **9**, 97-102.

Pacella E., La Torre G., De Giusti M., et al. (2013). Results of case-control studies support the association between contact lens use and *Acanthamoeba* keratitis. *Clin Ophthalmol*. **7**, 991-914.

Pinna A., Porcu T., Boscia F., Cano A., Erre G., et al. (2017). Free-Living Amoebae Keratitis. *Cornea*. **36**, 785-790.

Posada D. (2009). Selection of models of DNA evolution with jModelTest, *Methods Mol Biol.* **537**, 93-112.

Qvarnstrom Y., Visvesvara G.S., Sriram R., da Silva AJ. (2006). Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri. J Clin Microbiol*, **44**, 3589-3595.

Rivière D., Szczebara F.M., Berjeaud J.M., Frère J., Héchard Y. (2006). Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. *J Microbiol Methods*. **64**, 78-83.

Schroeder J.M., Booton G.C., Hay J., Niszl I.A., Seal D.V., et al. (2001). Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoebae* from humans with keratitis and from sewage sludge. *J Clin Microbiol.* **39**, 1903-1911.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol.* **30**, 2725-2729.