

Isolation and genotyping of *Acanthamoeba* strains from corneal infections in Italy

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Acanthamoeba keratitis (AK) is a corneal disease caused by members of a genus of free-living amoebae and is associated predominantly with contact lens (CL) use. This study reports 16 cases of culture-proven AK diagnosed in northern Italy. Genotype identification was carried out with a PCR assay based on sequence analysis of the 18S rRNA gene, and sensitivity and specificity were evaluated in comparison with traditional parasitological techniques. A 405 bp region of the 18S rRNA gene (ASA.S1) including diagnostic fragment 3 (DF3) was amplified using the genus-specific primers JDP1 and JDP2. Genotype assignment was based on phenetic analysis of the ASA.S1 subset of the nuclear small-subunit rRNA gene sequence excluding the highly variable DF3 region. Phylogenetic analysis was also performed on the sequences obtained. All patients complained of monolateral infection; 11 (68.75%) admitted improper CL disinfection. In 14/16 (87.5%) subjects, corneal scrapings were stained with calcofluor white and haematoxylin and eosin and, in ten cases (62.5%), microscopy was positive for *Acanthamoeba* cysts. *In vitro* culture on 3% non-nutrient agar plates was obtained in all cases (100%), whereas cloning and axenic growth were positive for 14 amoebic stocks (87.5%). PCR analysis had 100% sensitivity and specificity compared with *in vitro* axenic culture, showing positive amplification from 15 isolates. All *Acanthamoeba* strains belonged to the T4 genotype, the main AK-related genotype worldwide. These results confirmed the importance of a complete diagnostic protocol, including a PCR assay, for the clinical diagnosis of AK on biological samples. Genotyping allowed inclusion of all isolates in the T4 group, thus demonstrating the prevalence of this genotype in northern Italy.

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INTRODUCTION

Acanthamoeba keratitis (AK) is a rare, but potentially blinding, corneal infection caused by ubiquitous free-living amoebae belonging to the genus *Acanthamoeba*. They have been isolated from soil, tap and recreational water, electric and nuclear power plant cooling towers, heating, ventilating and air-conditioning units, dental irrigation units, dialysis machines and dust, but also from bacterial, fungal

and mammalian cell cultures, contact-lens paraphernalia, ear discharge, pulmonary secretions and nasopharyngeal mucosa from both patients with respiratory complaints and healthy subjects (Visvesvara *et al.*, 2007).

AK occurs in immunocompetent subjects following corneal trauma or, more commonly, in contact-lens (CL) wearers as a result of poor hygiene (Visvesvara *et al.*, 2007). More than 5000 cases of AK had been reported in the USA as of August 2007, together with a number of cases in the UK and India (Sharma *et al.*, 2000; Seal, 2003). The disease is characterized by corneal inflammation, severe ocular pain, photophobia, central or paracentral stromal ring infiltrate and recurrent breakdown of the corneal epithelium. The

Abbreviations: AK, *Acanthamoeba* keratitis; CL, contact lens; CPE, cytopathic effect; DF3, diagnostic fragment 3; H&E, haematoxylin and eosin; HELF, human embryonic lung fibroblast; PAS, periodic acid-Schiff; PHMB, polyhexamethylene biguanide.

lesion, which is typically monolateral, is refractory to commonly used antibiotics and can provoke perforation and eventual vision loss (Marciano-Cabral & Cabral, 2003; Visvesvara *et al.*, 2007). Species differentiation is based on the study of isoenzyme profiles and molecular techniques. Isolated *Acanthamoeba* strains have been placed in three groups (I–III) based on biochemical assays carried out with high-resolution PAGE using three isoenzymes: hexokinase, esterase and acid phosphatase (Moura *et al.*, 1992).

Analysis of the 18S rRNA gene allows the differentiation of 15 genotypes (T1–T15) within the genus *Acanthamoeba* (Schuster & Visvesvara, 2004), of which genotype T4 appears to be the most common in the environment and in patients with AK (Booton *et al.*, 2005). T4 is also the major genotype associated with non-keratitis infections, such as granulomatous amoebic encephalitis and cutaneous infections (Khan, 2006). The large number of T4 isolates in human infections may be related to their virulence and/or properties that enhance their transmissibility, as well as their decreased susceptibility to chemotherapeutic agents (Maghsood *et al.*, 2005).

In the present study, we used a PCR analysis protocol to identify and characterize amoebic isolates from 16 human AK cases that occurred in Italian patients and to define the epidemiological distribution of *Acanthamoeba* genotypes in northern Italy.

METHODS

Clinical specimens. One sample (corneal scraping or corneal biopsy) from each of 16 patients wearing soft CLs was examined. In ten cases, the CLs were also cultured on non-nutrient (NN) agar plates enriched with *Escherichia coli* K12.

Microscopic diagnosis. The diagnostic protocol included: extemporaneous examination of calcofluor white-stained corneal scrapings; examination of haematoxylin and eosin (H&E)-stained or periodic acid–Schiff (PAS)-stained slides with corneal scrapings; *in vitro* culture of the same biological samples, CLs, cleaning solutions or fluid from CL storage on 3% NN agar plates coated with a layer of living *E. coli* K12 strain; cloning and growth of the amoebic strain in axenic media (Fulton and CGVS); and an *in vitro* pathogenicity assay on human embryonic lung fibroblast (HELFL) monolayers.

Molecular methods. Isolate DNA extractions from NN agar plates and from axenic cultures were performed using a QIAamp DNA Micro kit (Qiagen), according to the manufacturer's instructions, and stored at -20°C . A 405 bp region of the 18S rRNA gene (ASA.S1) that included diagnostic fragment 3 (DF3) was amplified using the genus-specific primers JDP1 and JDP2 (Schroeder *et al.*, 2001) in a final volume of 25 μl [12.5 μl $2 \times$ PCR master mix (Promega), 5 μl template DNA, 0.6 mM each primer]. The PCR cycle profile was as follows: initial denaturation at 96°C for 2 min, followed by 35 cycles of denaturation at 96°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. As a negative control for amplification (non-target), reaction mixtures containing only reagents and sterile distilled water were prepared and included simultaneously with the unknowns in each PCR run. Bands were visualized on SYBR Safe DNA-stained 1% agarose gels (Invitrogen). PCR products were purified using a

NucleoSpin Extract purification kit (Macherey-Nagel) and sequenced on both strands by Bio-Fab Research (<http://www.biofabresearch.it/>).

Genotype assignment was based on phenetic analysis of the ASA.S1 subset of the nuclear small-subunit rRNA gene sequence excluding the highly variable DF3 region. Nucleotide sequences of the homologous gene fragments from all genotypes were retrieved from GenBank (see Fig. 1 for accession numbers). Phylogenetic analysis based on the sequences obtained was performed using MEGA (version 4.0). Distance-based analyses were conducted using the maximum composite likelihood method, and trees were constructed using a neighbour-joining algorithm. Bootstrap proportions were calculated by analysing 500 replicates.

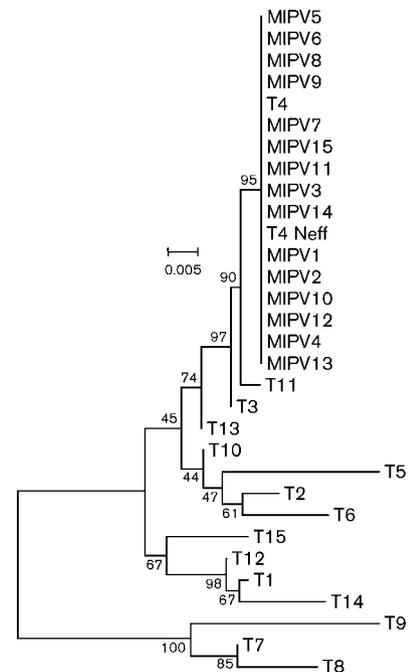


Fig. 1. Phylogenetic relationships of *Acanthamoeba* samples inferred by neighbour-joining analysis of the 18S rRNA gene sequence excluding the DF3 region using maximum composite likelihood distance estimates with 500 bootstrap replications and produced in MEGA 4.0. Abbreviations for the Italian isolates are defined in Table 1. The GenBank accession numbers and the origins of the isolates used for comparison are: T1, *Acanthamoeba castellanii* CDC:0981:V006 (GenBank accession no. U07400); T2, *Acanthamoeba palestinensis* Reich ATCC 30870 (AF479563); T3, *Acanthamoeba griffini* S-7 ATCC 30731 (U07412); T4 Neff, *A. castellanii* Neff ATCC 50373 (U07416); T4, *Acanthamoeba* sp. S27 (DQ087323); T5, *Acanthamoeba lenticulata* PD2S (U94741); T6, *A. palestinensis* 2802 (AF019063); T7, *Acanthamoeba astronyxis* CCAP 1534/1 (AF239293); T8, *Acanthamoeba tubiashi* OC-15C (AF019065); T9, *Acanthamoeba comandoni* Comandon & de Fonbrune (AF019066); T10, *Acanthamoeba* sp. CDC # V369 (AY703001); T11, *Acanthamoeba* sp. PN14 (AF333608); T12, *Acanthamoeba healyi* CDC 1283:V013 (AF019070); T13, *Acanthamoeba* sp. UWC9 (AF132134); T14, *Acanthamoeba* sp. PN15 (AF333607); T15, *Acanthamoeba jacobsi* AC305 (AY262365). Bar, 0.005 nucleotide substitutions per site. Numbers below nodes represent percentage bootstrap values.

Statistical analysis. To assess the association between clinical severity and either delay before AK diagnosis or prevalence of a positive *in vitro* cytopathic effect (CPE) of the amoebic isolates on HELF monolayers, Kruskal–Wallis and Fisher's exact tests were used, respectively. Continuous data were described as the median and 25th–75th percentiles, categorical variables as counts and percentages. Medcalc version 11 (Medcalc Software) was used for computation. A two-sided *P* value of <0.05 was considered statistically significant.

RESULTS

Descriptive epidemiology

Patient epidemiology is shown in Table 1. Sixteen patients were examined [ten females (62.5%); six males (37.5%); median age 37.7 years, range 21–60 years) with monolateral infection [left eye, nine subjects (56.25%); right eye, seven subjects (43.75%)]. All subjects used CL solutions [seven (43.7%) used solutions containing 3% hydrogen peroxide, nine (56.3%) used solutions containing 0.0001% (w/v) polyhexanide], but 11 (68.7%) admitted occasional improper CL disinfection with tap water.

In Table 2, the clinical features, therapy and outcome of the series are reported. Initial diagnoses were: herpetic keratitis in six cases (37.5%), bacterial/mycotic infection in two cases (12.5%), corneal ulceration in two cases (12.5%), conjunctivitis in one case (6.25%), keratoendothelitis due to CL in one case (6.25%) and non-specified keratitis in three cases (18.75%). In one case (6.25%), corneal rejection after transplantation was recorded. Steroid therapy was initially administered in 14 patients (87.5%). The median delay time between initial diagnosis and *Acanthamoeba* diagnosis was 9.5 weeks (range 1–32 weeks).

The most common clinical features at diagnosis were: corneal ring infiltrate (seven cases, 43.75%), diffuse corneal infiltrate (four cases, 25.0%), central ulcer with hypopyon (one case, 6.25%), severe uveitis (one case, 6.25%), corneal perforation (one case, 6.25%), subepithelial and perineural infiltrates (one case, 6.25%) and central epithelium opacity (one case, 6.25%).

Clinical status and therapy

In order to establish a correlation between delay in diagnosis and treatment and severity of the disease at presentation, we arbitrarily divided the patients into three groups: (i) mild disease, in cases of corneal infiltrates without intraocular involvement (five patients); (ii) moderate disease, for corneal ulcers without intraocular involvement (two patients); and (iii) severe disease, when corneal infiltrates and/or ulcers were associated with intraocular involvement (endothelial infiltrates, perforation, iris retraction, uveitis) (nine patients).

Treatment was based on a combination of 0.02% polyhexamethylene biguanide (PHMB) with Desomedine in eight patients (50.0%) and 0.02% chlorhexidine with Desomedine in eight cases (50%) and, in five patients (31.25%), PHMB was switched to chlorhexidine because of a toxic reaction. In all cases, the infection was successfully treated. At the end of the treatment, best spectacle-corrected visual acuity was better than 0.6, without any surgery, in seven patients (43.75%). Three patients (18.75%) underwent penetrating keratoplasty 'à chaud' for impending risk of perforation, two (12.5%) underwent optical penetrating keratoplasty, three (18.75%) had

Table 1. Epidemiological and parasitological data for the 16 Italian patients with AK

F, Female; M, male; L, left; R, right; PHX, 0.0001% polyhexanide; HP, 3% hydrogen peroxide; ND, not done; NR, not reported.

Patient	Strain no.	Age (years)	Sex	Infected eye	Use of tap water	CL solution	Calcofluor white staining	H&E or PAS staining	3% NN agar	Axenic culture	CPE on HELFs	PCR	Genotype
1	MIPV-3	37	F	L	Yes	PHX	+	+	+	+	–	+	T4
2	MIPV-4	26	F	R	Yes	HP	–	–	+	–	ND	+	T4
3	MIPV-2	60	M	R	Yes	PHX	+	+	+	+	+	+	T4
4	MIPV-7	27	F	L	Yes	HP	+	+	+	+	–	+	T4
5	MIPV-8	27	M	L	Yes	PHX	+	+	+	+	+	+	T4
6	MIPV-11	36	F	L	Yes	HP	+	+	+	+	+	+	T4
7	MIPV-13	54	F	L	NR	PHX	ND	ND	+	+	–	+	T4
8	MIPV-1	51	M	L	Yes	HP	+	+	+	+	+	+	T4
9	MIPV-6	21	F	R	Yes	PHX	–	–	+	+	+	+	T4
10	MIPV-16	28	M	L	Yes	HP	–	–	+	–	ND	ND	ND
11	MIPV-14	58	F	R	Yes	PHX	+	+	+	+	–	+	T4
12	MIPV-15	40	F	L	NR	HP	–	–	+	+	–	+	T4
13	MIPV-9	31	M	R	NR	PHX	ND	ND	+	+	+	+	T4
14	MIPV-12	22	F	R	No	PHX	+	+	+	+	–	+	T4
15	MIPV-5	31	F	R	Yes	HP	+	+	+	+	–	+	T4
16	MIPV-10	55	M	L	NR	PHX	+	+	+	+	–	+	T4

Table 2. Clinical and therapeutic data for the 16 Italian patients with AK

BCVA, Best corrected visual acuity; HM, hand motion; LK, lamellar keratoplasty; PK, penetrating keratoplasty; PTK, excimer laser phototherapeutic keratectomy.

Patient	Strain no.	First diagnosis	Steroid therapy	Delay before AK diagnosis (days)	Severity	PHMB	Desomedine	Chlorhexidine	Corneal graft	BCVA	Complications
1	MIPV-3	Bacterial infection	Yes	58	Severe	Yes	Yes	Yes	PK 'à chaud'	0.7	Cataract: waiting
2	MIPV-4	Herpetic keratitis	Yes	39	Mild		Yes	Yes	No	1.0	Cataract: operated
3	MIPV-2	Corneal ulcer	No	11	Severe		Yes	Yes	No	0.8	
4	MIPV-7	Herpetic keratitis	Yes	130	Moderate	Yes	Yes	Yes	Optical PK	0.9	
5	MIPV-8	Bacterial-mycotic infection	Yes	95	Severe		Yes	Yes	No	0.9	
6	MIPV-11	Herpetic keratitis	No	70	Mild	Yes	Yes		No	0.3	Waiting for optical LK
7	MIPV-13	Keratitis	Yes	73	Severe		Yes	Yes	PK 'à chaud'	HM	Severe glaucoma
8	MIPV-1	CL keratoendothelitis	Yes	31	Severe	Yes	Yes		Tectonic LK	0.1	Waiting for optical PK
9	MIPV-6	AK + herpetic uveitis	Yes	58	Severe	Yes	Yes		PTK	0.8	
10	MIPV-16	Conjunctivitis	Yes	18	Moderate		Yes	Yes	Optical LK	0.9	
11	MIPV-14	Keratitis	Yes	42	Severe	Yes	Yes	Yes	Optical LK	HM	Severe glaucoma
12	MIPV-15	Herpetic keratitis	Yes	258	Mild	Yes	Yes	Yes	No	0.4	Waiting for optical LK
13	MIPV-9	Keratitis	Yes	101	Severe	Yes	Yes	Yes	PK 'à chaud'	HM	Severe glaucoma
14	MIPV-12	Herpetic keratitis	Yes	24	Mild		Yes	Yes	No	0.9	
15	MIPV-5	Corneal ulcer, hypopyon	Yes	17	Mild		Yes	Yes	No	0.7	
16	MIPV-10	Herpetic keratitis; corneal rejection	Yes	45	Severe	Yes	Yes	Yes	Optical PK	0.7	Waiting for optical LK

optical or tectonic lamellar keratoplasty and four patients (25.0%) were waiting for surgery. In three cases (18.75%), visual acuity remained very poor because of optic nerve damage due to severe secondary glaucoma (hand motion only; Table 2).

Parasitological diagnosis

A single specimen (corneal scraping or biopsy) was examined for each subject. In 14/16 patients (87.5%), calcofluor white- and H&E-stained slides were produced from corneal scrapings. In ten cases (62.5%), microscopic examination identified *Acanthamoeba* cysts (Table 1). *In vitro* culture in 3% NN agar plates allowed strain isolation in all 16 cases (100%) although, in one case (patient 2), the growth of the amoebic strain was very difficult and it was eventually lost. Subsequent isolate cloning and growth in axenic liquid medium was positive in 14 of the 15 remaining amoebic stocks (87.5%). The pathogenicity assay on HELF monolayers showed a CPE after 24–48 h of contact for six (37.5%) amoebic isolates whereas, in eight cases (50%), only adhesion of the *Acanthamoeba* trophozoites to the fibroblasts was observed (Table 1). In six cases (37.5%), strain isolation was also carried out from patient CL culture.

Microscopic studies of the morphological characteristics of the cysts allowed us to include the amoebic isolates in group II of the Pussard–Pons classification (Pussard & Pons, 1977).

Molecular analysis

Successful PCR amplification was obtained from all 15 amoeba-positive stocks. Sequence analysis using a BLAST search indicated an identity of >98% with *Acanthamoeba* 18S rRNA gene reference sequences.

Fig. 1 shows the phylogenetic tree obtained by neighbour-joining analysis of ASA.S1 excluding DF3 sequences from the different *Acanthamoeba* genotypes. All *Acanthamoeba* isolates investigated in this study formed a well-defined cluster including the two T4 reference sequences and supported by significant bootstrap values, whereas all other genotypes were grouped in various subgroups. Thus, genotype T4 showed itself to be the only genotype responsible for AK in our clinical cases; this agrees with the results of previous studies (Booton *et al.*, 2005; Khan, 2006; Di Cave *et al.*, 2009).

Association with clinical findings

Because of the small number of patients, for the purpose of this analysis we collapsed patients with ‘moderate’ infection (two patients) with the patients with ‘severe’ disease. The median delay in diagnosis was higher, but not significantly (Kruskal–Wallis test, $P=0.69$), in the moderate to severe disease category than in the mild disease category [58 (34–89) vs 39 (22–117)]. Three of the four

cases with mild disease (75%) compared with six of the ten cases with moderate to severe disease (60%) had a positive *in vitro* CPE on HELFs, with no significant difference using Fisher’s exact test ($P=1.0$).

DISCUSSION

Acanthamoeba strains are said to be responsible for up to 20% of infectious keratitis in CL wearers (McAllum *et al.*, 2009); to date, more than 5000 cases have occurred in the USA (Visvesvara *et al.*, 2007) and large numbers of amoebic infections have also been reported from the UK, Europe, India and Hong Kong (Seal, 2003).

Clinical diagnosis is difficult, and the disease is often mistaken for herpes simplex, adenovirus or fungal keratitis (Hammersmith, 2006), as is laboratory diagnosis, where about 60% of cases are misdiagnosed using direct detection techniques (Yera *et al.*, 2007), primarily because of the inability of pathologists and laboratory technicians to recognize these pathogens morphologically in biological samples (Qvarnstrom *et al.*, 2006).

From the epidemiological point of view, our study confirmed the risk of irregular or inadequate disinfection caused by CL case cleaning with tap water or by exposure to contaminated water (Stehr–Green *et al.*, 1987; Cohen, 2009). In fact, 11 patients (68.75%) admitted improper disinfection of their CLs with tap water, although they also used marketed CL solutions, such as 3% hydrogen peroxide or 0.0001% polyhexanide solutions. Hydrogen peroxide is known to be very effective at CL disinfection due to its ability to destroy bacteria, fungi and *Acanthamoeba* strains by oxidation (Joslin *et al.*, 2007; Johnston *et al.*, 2009; Verani *et al.*, 2009). In contrast, solutions without hydrogen peroxide show varying degrees of activity against *Acanthamoeba* strains (Johnston *et al.*, 2009). In our series, data on the anti-protozoan activity of the CL disinfecting solutions used by the patients were not recorded.

Presumably, the *Acanthamoeba* portal of entry for the other subjects was represented by corneal abrasions due to CL wearing (Alizadeh *et al.*, 2005). CL wear is also associated with upregulation of mannose-6-phosphate receptor proteins located on the epithelial corneal surface, which increases the amoeba’s ability to destroy tissue by producing epithelial apoptosis mediators and proteases (Alizadeh *et al.*, 2005; Khan, 2006).

The delay in establishing the correct amoebic aetiology of the infections (a median of 9.5 weeks; range 1–32 weeks), along with the administration of steroid therapy in the majority of our patients (14/16; 87.5%), caused appreciable worsening in clinical presentation and resulted in two cases of corneal ulceration. Although steroids are prescribed to reduce inflammation and pain, they can simultaneously increase the pathogenicity of the amoebae by suppressing the patient’s immune response (Lindsay *et al.*, 2007; Khan, 2009).

Possibly because of the rarity of AK, we were not able to show an association between the severity of the disease and either delay in diagnosis or an *in vitro* CPE on HELFs by amoebic isolates, although the delay appeared to be shorter in milder cases.

As evidence of the importance of collaboration between clinicians and laboratory technicians, correct diagnosis of amoebic infection was achieved using parasitological methods that allowed direct microscopic identification of the pathogens (10/16; 62.5%) and/or *in vitro* isolation of the strain (16/16; 100%). In agreement with other authors (Schuster, 2002; Seal, 2003; Khan, 2009), we also recognize that *Acanthamoeba* culture from biological specimens is the best assay for these organisms. However, production of an adequate amoebic population can take a week or longer. In addition, strain axenization requires the use of complex liquid media containing peptone, yeast extract, glucose and fetal calf serum, and it is sometimes impossible to eradicate the accompanying bacterial or fungal flora, as in one of our cases (Table 1, patient 10).

In the rest of our series (14/16; 87.5%), we tested the pathogenicity of the *Acanthamoeba* isolates on HELF monolayers and observed a CPE in 24–48 h for six strains (37.5%). In the remaining eight, only close trophozoite adherence to the fibroblasts was reported. One explanation for this feature might be the different degrees of virulence of the isolates (Khan, 2009), although no significant association could be elicited between a marked phagocytic activity and clinical severity.

All the amoebic strains were included in group II of the Pussard–Pons classification (Pussard & Pons, 1977) based on the morphological features of the cystic forms. This group includes the most widespread and commonly isolated *Acanthamoeba*. These data were confirmed by molecular analysis; in fact, by analysing the 18S rRNA gene sequence, it was shown that all of the isolates belonged to the T4 genotype, which appears to be the most common form in northern Italy, in addition to being the most prevalent form related to keratitis infection worldwide (Khan, 2009; Zhao *et al.*, 2010).

Analysis of phylogenetic relationships within the genus was useful in characterizing the *Acanthamoeba* strains in this study. Although not reported, we also analysed the tree produced by including the entire sequence (including the DF3 region). The tree topology was very similar to that obtained for the dataset sequence excluding DF3. In the latter case, genotypes could be better distinguished by high bootstrap values or distance of separation, thus highlighting that the DF3 region is a target to detect intragenotype variation, rather than a tool for genotype assignment, as reported previously (Di Cave *et al.*, 2009; Ledee *et al.*, 2009).

A PCR assay, which is more rapid and sensitive than traditional parasitological techniques, permits certain aetiological diagnoses and therefore prompts specific

therapy to enhance the chances of complete recovery (Visvesvara *et al.*, 2007). In addition, integration of a PCR method with the conventional parasitological techniques of smear examination and *in vitro* culture may complete the diagnostic protocol in the case of AK and offer information on the pathogenicity of the amoebic isolates (Qvarnstrom *et al.*, 2006; Khan, 2009).

The mainstay of treatment for AK, as reported in our study, remains the combination of a topical biguanide, such as PHMB or chlorhexidine, plus topical diamidine, such as propamidine or hexamidine (Seal, 2003). The main antimicrobial effect of biguanides occurs through interaction with the lipid bilayer of the amoebal plasma membrane causing membrane damage and pore formation leading to cell lysis (Stapleton *et al.*, 2009). Aromatic diamidines cause membrane damage too and inhibit polyamine metabolism by intercalating with amoebic DNA (Seal, 2003).

AK is an emerging infectious disease and delayed or misdiagnosis can result in permanent corneal damage and even blindness. Wearing CLs is a significant risk factor in this infection, and users should be aware of the risks resulting from improper hygiene procedures. Good CL care compliance also reduces the risk of other, more common, forms of microbial keratitis.

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