The potential pathogenicity of chlorhexidine-sensitive *Acanthamoeba* strains isolated from contact lens cases from asymptomatic individuals in Tenerife, Canary Islands, Spain

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Pathogenic strains of the genus *Acanthamoeba* are causative agents of a serious sight-threatening infection of the eye known as *Acanthamoeba* keratitis. The prevalence of this infection has risen in the past 20 years, mainly due to the increase in number of contact lens wearers. In this study, the prevalence of *Acanthamoeba* in a risk group constituted by asymptomatic contact lens wearers from Tenerife, Canary Islands, Spain, was evaluated. Contact lenses and contact lens cases were analysed for the presence of *Acanthamoeba* isolates. The isolates' genotypes were also determined after rDNA sequencing. The pathogenic potential of the isolated strains was subsequently established using previously described molecular and biochemical assays, which allowed the selection of three strains with high pathogenic potential. Furthermore, the sensitivity of these isolates against two standard drugs, ciprofloxacin and chlorhexidine, was analysed. As the three selected strains were sensitive to chlorhexidine, its activity and IC₅₀ were evaluated. Chlorhexidine was found to be active against these strains and the obtained IC₅₀ values were compared to the concentrations of this drug present in contact lens maintenance solutions. It was observed that the measured IC₅₀ was higher than the concentration found in these maintenance solutions. Therefore, the ineffectiveness of chlorhexidine-containing contact lens maintenance solutions against potentially pathogenic strains of *Acanthamoeba* is demonstrated in this study.

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INTRODUCTION

Free-living amoebae of the genus *Acanthamoeba* are ubiquitous protozoans that pervade the entire environment and include amphizoic strains that are pathogenic to humans and animals (Schuster & Visvesvara, 2004a). These protozoans are opportunistic causal agents of a sight-threatening ulceration of the cornea called *Acanthamoeba* keratitis (AK), disseminated infections (mostly cutaneous and nasopharyngeal) and a usually fatal granulomatous amoebic encephalitis (Khan, 2003, 2006; Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004a).

AK in developed countries is often associated with inadequate care of the contact lenses and also as a consequence of corneal trauma whereas in developing

nations most cases occur as a result of ocular trauma (Seal *et al.*, 1999; Ibrahim *et al.*, 2007; Ozkoc *et al.*, 2008). AK symptoms are nonspecific and can be misdiagnosed as a viral, bacterial or fungal keratitis. Thus an early diagnosis is required to achieve a successful therapeutic outcome (Martínez & Visvesvara, 1991; Lorenzo-Morales *et al.*, 2007). Currently, the recommended treatment regimen includes a biguanide [0.02 % polyhexamethylene biguanide (PHMB) or 0.02 % chlorhexidine digluconate] together with a diamidine (0.1 % propamidine isethionate, also known as Brolene, or 0.1 % hexamidine, also known as Desomedine) (Khan, 2006). Biguanides are most often used due to their excellence in the treatment of AK and are frequently combined with a diamidine due to their presumed additive anti-amoebic effect (Hay *et al.*, 1994; Schuster & Visvesvara, 2004b; Lim *et al.*, 2008). Moreover, chlorhexidine and PHMB as monotherapy agents at normal concentration have been proven not to be sufficient against clinical or environmental strains of *acanthamoebae* and this has highlighted the importance of multiple-strain testing of drugs against *acanthamoebae* as their effective-

Abbreviations: AK, *Acanthamoeba* keratitis; PHMB, polyhexamethylene biguanide.

The GenBank/EMBL/DDBJ accession numbers for the diagnostic fragment 3 sequence of the 28 new *Acanthamoeba* isolates are EU686695–EU686722.

ness might depend on the *Acanthamoeba* isolate (Lim *et al.*, 2008; Gooi *et al.*, 2008; Shoff *et al.*, 2008).

It is also important to mention that drugs to be used for the treatment of AK should be both amoebicidal and cysticidal as there is a risk of relapse due to the re-emergence of amoebae from the more resistant cyst (Schuster & Visvesvara, 2004b). Chlorhexidine has been shown to be active against both *Acanthamoeba* trophozoites and cysts, as it interferes with the mucopolysaccharides of the cyst at the ostiole level and also in the lipid bilayer of the cellular membrane, causing cellular lysis (Lim *et al.*, 2008).

The taxonomy and classification of these protozoans are continually under revision, following the successful application of molecular techniques (Stothard *et al.*, 1998; Booton *et al.*, 2002, 2005). Evolutionary studies have led to the identification of 15 different genotypes (T1–T15) based on rRNA gene sequencing (Stothard *et al.*, 1998; Horn *et al.*, 1999; Gast, 2001; Hewett *et al.*, 2003). The correlation between pathogenicity and certain genotypes continues to be investigated (Stothard *et al.*, 1998; Gast, 2001; Booton *et al.*, 2005; Maghsood *et al.*, 2005). To date, studies have shown that 90 % of *Acanthamoeba* isolates that produce infections belong to the T4 genotype, suggesting that the abundance of T4 isolates in human infections is most likely due to their greater virulence and/or properties that enhance their transmissibility as well as their decreased susceptibility to chemotherapeutic agents (Maghsood *et al.*, 2005; Khan, 2006).

A role for extracellular proteases, mainly serine, metallo- and cysteine proteases, in the pathogenicity of *Acanthamoeba* has been suggested previously (Khan, 2003), and higher quantities of extracellular proteases from pathogenic compared to non-pathogenic strains seem to be responsible for epithelial-cell destruction. Serine protease activity is likely to be a major factor in the pathogenesis of amoebic keratitis or granulomatous amoebic encephalitis consequential to *Acanthamoeba* infection (Lorenzo-Morales *et al.*, 2005a, c; Khan, 2006) as these proteases have shown an enhanced capacity to degrade proteins such as collagen, fibronectin, laminin, sIgA, IgG, plasminogen, fibrinogen and haemoglobin (Na *et al.*, 2001). It is important, therefore, to study the secretion of proteases as this allows the differentiation between pathogenic and non-pathogenic *Acanthamoeba* strains depending on the type, number and the degree of secretion (Khan, 2003; Lorenzo-Morales *et al.*, 2005a, c).

In this work, a number of different *Acanthamoeba* strains were isolated from contact lens cases from asymptomatic individuals in Tenerife, Canary Islands, Spain. Certain strains which were indicated to be particularly pathogenic by a number of criteria were further tested with respect to their sensitivity to chlorhexidine and ciprofloxacin. Chlorhexidine was demonstrated to be active against these strains, but the concentration of chlorhexidine required to kill these strains was higher than those found in commercial contact lens maintenance solutions.

METHODS

Isolation and cultivation. One hundred and fifty-three contact lens cases (90 of them included their contact lenses) from asymptomatic immunocompetent individuals from Tenerife, Canary Islands, Spain, were included in this study. Data regarding the type of contact lens, disinfectant systems, length of wear and contact lens hygiene were collected together with the contact lens cases and contact lenses.

Contact lens cases were washed using sterile saline solution. The obtained washings were filtered through a 0.45 µm pore diameter cellulose nitrate filter (Millipore) with a weak vacuum (flow rate 1.3 ml min⁻¹). These membranes were then cultured in 2 % non-nutrient agar (NNA) covered with heat-killed *Escherichia coli* and incubated at 28 °C to allow growth of non-pathogenic and pathogenic amoebal strains. After 3–4 days, the membranes were removed and the plates were incubated at 28 °C for a further 1–2 weeks. Swabs were then stroked on NNA plates within an area 40 by 40 mm in size and incubated at 28 °C for up to 2 weeks. These plates were monitored for out-growth of *Acanthamoeba* microscopically and blocks containing *Acanthamoeba* were removed and the amoebae were cloned by dilution. *Acanthamoeba* isolated in this way was then transferred into axenic cultures by placing the amoebae into PYG medium [0.75 % (w/v) proteose peptone, 0.75 % (w/v) yeast extract and 1.5 % (w/v) glucose] containing 40 µg gentamicin ml⁻¹ (Biochrom). Genus-specific PCR was carried out in order to confirm the positive cultures as previously described (Vodkin *et al.*, 1992).

Characterization of the pathogenic potential. The pathogenic potential of the isolated amoebae was evaluated using extracellular protease zymograms and assays to determine thermotolerance and osmotolerance, as previously described (Khan *et al.*, 2000; Lorenzo-Morales *et al.*, 2005c). Briefly, SDS-PAGE gels containing gelatin (2 mg ml⁻¹) were used for the analysis of extracellular proteases and the type of secreted protease was examined by using protease inhibitors such as PMSF (serine protease inhibitor, 2 mM final concentration), E-64 (cysteine protease inhibitor, 5 µM final concentration) or EDTA (metalloprotease inhibitor, 10 mM final concentration) for 30 min prior to electrophoresis.

For temperature tolerance assays, NNA plates seeded with *E. coli* were inoculated with approximately 1000 cells, and the plates were incubated at 28 °C, 37 °C and 44 °C for up to 72 h. To examine the effect of osmolarity on the growth of *Acanthamoeba* trophozoites, NNA plates containing 0.1 M, 1.0 M and 1.5 M mannitol were seeded with *E. coli* as previously described (Khan *et al.*, 2001). Approximately 1000 trophozoites were inoculated onto these plates and incubated at 28 °C for up to 72 h. Growth and the survival rate of amoebae were checked during this period and resistance to all assayed conditions classified the amoebae as thermo- and osmotolerant as described previously (Khan *et al.*, 2001).

Moreover, PCR of the serine protease catalytic domain (Hong *et al.*, 2000) was also performed as previously described as this assay together with the zymograms and thermo- and osmotolerance assays could determine the pathogenic potential of these amoebae (Lorenzo-Morales *et al.*, 2005b, c).

Activity assays. The activity of two drugs was tested on selected strains of *Acanthamoeba* from this study: chlorhexidine (chlorhexidine digluconate; Alfa Aesar), a standard antiseptic belonging to the biguanide family of compounds which are commonly used in contact lens maintenance solutions; and ciprofloxacin (ciprofloxacin lactate; Dr. Ehrenstorfer), an antibiotic that has been previously used for the treatment of AK (Colin & Hasle, 1993; Wynter-Allison *et al.*, 2005). For the sensitivity and activity assays, a type strain from the American Type Culture Collection, *Acanthamoeba castellanii* (Neff strain) ATCC 30010, genotype T4, was used as a control.

For the activity assays, a previously developed colorimetric 96-well microtitre plate assay, based on the oxidoreduction of alamarBlue, was used for the determination of drug efficacy against the trophozoites of the selected *Acanthamoeba* strains as previously described by McBride *et al.* (2005). Subsequently the plates were analysed, over a period of 72–120 h, on a Microplate Reader model 680 (Bio-Rad) using a test wavelength of 570 nm and a reference wavelength of 630 nm. For those strains that were sensitive to the assayed drugs, the percentage of inhibition and 50 % inhibitory concentrations (IC_{50}) were calculated by linear regression analysis using a 95 % confidence limit. All experiments were performed three times each in duplicate and the mean values were also calculated. A paired two-tailed *t*-test was used for analysis of the data. Values of $P < 0.05$ were considered significant. The inhibition curves of the statistical analysis were developed using the Sigma Plot 9.0 software program (Systat Software).

Genotyping of *Acanthamoeba* isolates. *Acanthamoeba* spp. genotype determination was based on sequence analysis of diagnostic fragment 3 (DF3), a subset of the nuclear small subunit rRNA gene as previously described. Obtained sequences were compared to sequences available in GenBank and genotypes of the new isolates were calculated by $<5\%$ dissimilarity as described previously (Stothard *et al.*, 1998; Booton *et al.*, 2002, 2005). Obtained sequences were also compared with the *Acanthamoeba* DNA sequence database (Department of Molecular Genetics, The Ohio State University, OH, USA). Sequences for the 28 new isolates are deposited in GenBank under the accession numbers EU686695–EU686722.

RESULTS

We found that 65.9 % of the analysed contact lenses and contact lens cases were positive for *Acanthamoeba*. Moreover, 30 % of the isolated amoebae were found to be potentially pathogenic and secreted extracellular serine proteases.

When the type of contact lens was evaluated, 81 % of the individuals in this study were monthly soft contact lens wearers. Furthermore, 52 % of the contact lenses were positive for *Acanthamoeba*, and 30 % of these amoebae were potentially pathogenic.

Regarding genotyping results, it was revealed that the isolated *Acanthamoeba* strains belonged to the T1 (3.6 %), T3 (14.3 %), T4 (78.6 %) and T10 (3.6 %) genotypes.

The most common maintenance solution used by 49 % of the study sample was the so-called 'one-step' system. This finding was important, as 52 % of the 'one-step' users were positive for *Acanthamoeba*, and 28 % of the strains isolated were determined to be potentially pathogenic (Table 1). If the antiseptic content of the maintenance solutions is considered (Fig. 1), 65 % of the individuals in this study were using solutions containing PHMB. Of the latter group, 48 % were positive for *Acanthamoeba* strains, of which 26 % were potentially pathogenic strains.

Zymograms of extracellular proteases showed that all the isolated amoebae were protease secretors. Additionally, 30 % of the isolates secreted serine proteases, as determined by zymography and PCR amplification of the serine protease catalytic domain, and therefore were considered

Table 1. Percentages of use, lenses positive for *Acanthamoeba* in each group and percentage of potentially pathogenic strains related to the number of positives found in this work

Data are shown for each type of contact lens included in this study.

Type of lens	Use (%)	Positives (%)	Potentially pathogenic (%)
Hard	0	0	0
Daily soft	7	37	0
Monthly soft	81	52	30
Bi-monthly soft	3	67	50
One-year soft	4	50	0
Two-year soft	5	50	33

potentially pathogenic. Based on the zymograms and PCR of the serine protease catalytic domain as well as genotyping results, three strains were selected for the sensitivity and activity assays mentioned previously: CLC-16 (T3 genotype), CLC-41.r (T4 genotype) and CLC-51.l (T1 genotype). Moreover, most of the proteases that were secreted by these strains were serine proteases. In the case of CLC-51.l, metalloproteases were also observed (Fig. 2).

All tested strains, CLC-16, CLC-41.r, CLC-51.l and *A. castellanii* Neff, were sensitive to ciprofloxacin and chlorhexidine assayed microscopically and colorimetrically. We chose to study the effect of chlorhexidine as it belongs to a family of molecules that are common components of contact lens maintenance solutions. Activity assays with

Fig. 1. Percentages of use for different antiseptics in the maintenance solutions of the contact lens wearers included in this study. The most common antiseptic was PHMB. % Use=% of contact lens wearers using each type of contact lens; % positives=% of these cases where *Acanthamoeba* was found; % pathogens=% of these *Acanthamoeba* strains that were determined to be likely pathogens through protease and osmo-tolerance assays.

Fig. 2. Protease zymograms of CLC-51.l incubated with different protease inhibitors (PMSF for serine proteases, E-64 for cysteine proteases and EDTA for metalloproteases). Most of the secreted proteases were serine proteases, although evidence of a metalloprotease was also observed, EDTA lane (arrow).

chlorhexidine and the IC_{50} data for each strain indicated that this compound was able to inhibit the growth of these amoebae even at low concentrations. The IC_{50} values at 96 h were 11.89 ± 3.80 , 2.45 ± 0.20 , 10.47 ± 1.48 and 5.23 ± 0.55 μ M for CLC-16, CLC-41.r, CLC-51.l and the type strain *A. castellanii* Neff, respectively.

DISCUSSION

Over the last few decades, AK has become increasingly recognized as important in human health. With an increasing number of people wearing contact lenses, it is important to assess any associated risks, and to make both existing and new users aware of these risks (Khan, 2006). In this study, the number of contact lens wearers that were positive for *Acanthamoeba* was much higher than in other studies. In a smaller study based in the south-west of Scotland, UK, *Acanthamoeba* were isolated from 46 (2.2 %) contact lens storage cases tested (Seal *et al.*, 1999). A previous larger study found *Acanthamoeba* in 4 out of 178 (2.2 %) lens cases from asymptomatic users in the same region (Devonshire *et al.*, 1993). The reason for the large difference in number is presently unknown but may be connected to the warm climate and dust encountered in Tenerife.

The AK incidence rate also varies between different geographical locations, and these variations are most likely related to extended wear of soft contact lenses, lack of awareness of the potential risks associated with wearing contact lenses, enhanced detection and/or local conditions that promote growth of pathogenic amoebae such as water hardness or salinity (Seal *et al.*, 1999; Radford *et al.*, 2002; Khan, 2006). The latter is highly important in relation to this study as potentially pathogenic strains of *Acanthamoeba*, including strains belonging to T4 genotype, were previously isolated from soil and water sources on the island of Tenerife (Lorenzo-Morales *et al.*, 2005b). We

found that 78.6 % of the strains isolated in this study belonged to genotype T4, confirming previous reports that T4 is the most common genotype worldwide (Khan, 2006).

The most commonly used antiseptics in contact lens maintenance solutions are PHMB (at concentrations of between 0.0001 % and 0.002 %) and hydrogen peroxide (maximum 3 %). However, most solutions also contain hydrant components such as sodium hyaluronate and antifungal agents.

It is also important to mention that PHMB and chlorhexidine (both biguanides) have shown amoebicidal and cysticidal properties against AK isolates and various type strains of *Acanthamoeba* (Larkin *et al.*, 1992; Chomicz *et al.*, 2005), and that the clinical outcome is similar for both (Lim *et al.*, 2008). In this study, a high number of users of biguanide-containing (mostly PHMB) maintenance solutions were positive for *Acanthamoeba*. This could be explained if the obtained IC_{50} values of chlorhexidine with the strains tested in this study are compared to the concentrations of biguanides (mostly PHMB) present in most maintenance solutions, these being much lower than the concentrations used in this study. The concentrations of biguanides (mostly PHMB) in maintenance solutions are not sufficiently high enough to destroy all amoebic contaminants. Previous studies have demonstrated that disinfectant systems are only effective if recommended hygiene regimes are strictly followed (Borazjani & Kilvington, 2005). It is also important to mention recent reports regarding the existence of different T4 strains that have shown diverse resistance levels against anti-amoebic drugs (Shoff *et al.*, 2007). Therefore, multiple-strain testing of drugs against *acanthamoebae* is recommended as their effectiveness might depend on the isolate (Shoff *et al.*, 2007; Lim *et al.*, 2008; Gooi *et al.*, 2008).

Another important observation in this study was the existence of variations in the incidence of potentially pathogenic *Acanthamoeba* strains depending on the type of contact lenses and length of use. As suggested above, the major risk factor for AK is poor lens hygiene (Schuster & Visvesvara, 2004a; Khan, 2006). In support of this statement, more than 85 % of AK cases occur in wearers of contact lenses and this fact is associated with individual behaviour regarding poor personal hygiene, poor handling and care of their lenses or lens storage cases, and non-compliance with disinfection procedures such as using home-made saline or contact with contaminated water sources (Niederhorn *et al.*, 1999; Brennan, 2002; Khan, 2006). These observations were corroborated by the results obtained in this study, as no potentially pathogenic amoebae were isolated from any daily disposable soft contact lens. In contrast, a high number of potentially pathogenic strains were isolated from soft contact lenses worn monthly or bi-monthly. With regards to the monthly use of soft contact lenses, the increased presence of amoebae may be explained by the overuse of the 'one-step' system and the general misuse and the poor hygiene

adopted for these types of lenses and their cases. It is also important to mention previous observations regarding the lack of efficacy against *acanthamoebae* of the majority of these one-step solutions (Hiti *et al.*, 2002, 2005; Mowrey-McKee & George, 2007). This risk of poor hygiene and care is obviously increased in the case of contact lenses worn bi-monthly as confirmed by the results in this study (Table 1). Finally, the 1-year- or 2-year-use contact lenses showed similar percentages for the presence of *acanthamoebae*; however, potentially pathogenic *Acanthamoeba* strains were only isolated from the 2-year-use contact lenses. In the case of 1-year-use lenses, this could be due to the recommendations of complementary care such as 'enzymatic' treatments that commonly contained hydrogen peroxide. Two-year-use contact lenses presented a high percentage of potentially pathogenic amoebae mostly due to a lack of hygiene and poor care of the lenses and their cases (Table 1).

The strains selected for the sensitivity and activity assays were more sensitive to chlorhexidine than to ciprofloxacin. Even so, ciprofloxacin should be considered for a combined therapy as antibiotics can reduce bacterial infection and also eliminate a source of nutrients for *acanthamoebae* (Khan, 2006). Furthermore, chlorhexidine would be a good candidate for inclusion in contact lens maintenance solutions as it is an inexpensive antiseptic that has been classified as an essential drug by the World Health Organization. Moreover, as previously mentioned, chlorhexidine is active against both trophozoites and cysts of *Acanthamoeba*, even at minimal concentrations that are not toxic to corneal epithelium cells (Green *et al.*, 1980; Lee *et al.*, 2007).

In conclusion, chlorhexidine, rather than PHMB, or other biguanides should be considered for inclusion in contact lens maintenance solutions, especially in light of the fact that PHMB has been reported to be cytotoxic to human corneal cells (Lee *et al.*, 2007). Additionally, the concentration of chlorhexidine or other biguanides should be re-evaluated as the maintenance solutions were shown to be ineffective against the potentially pathogenic strains isolated during this study.

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