Eye (2003) 17, 893–905

www.nature.com/eye

Acanthamoeba keratitis update—incidence, molecular epidemiology and new drugs for treatment

DV Seal

Abstract

A reliable figure for the expected incidence of *Acanthamoeba* keratitis of one per 30 000 contact lens wearers per year has now been obtained from a combination of three cohort and three Questionnaire Reporting Surveys; 88% of cases wore hydrogel lenses and 12% wore rigid lenses. This figure now provides a basis for the expected number of cases against which to judge either epidemic outbreaks or effects of prevention with disinfecting solutions, better hygiene, or the use of disposable lenses. Molecular biology of *Acanthamoeba* has advanced considerably in the last 10 years with new automated sequencing technology. This has allowed the construction of a genotype identification scheme with 13 different genotypes against which to compare clinical isolates for epidemiological investigations or pathogenicity markers. So far, only four genotypes have been associated with keratitis of which the majority have been T4 but T3, T6, and T11 have each caused individual cases. Each genotype is heterogenous and can be further subdivided by comparison of sequences of diagnostic fragments of 18S rDNA, riboprinting by PCR-RFLP of 18S rDNA, or by mitochondrial DNA RFLP. Drug therapy has been revolutionised with the introduction of the biguanides—chlorhexidine or polyhexamethylene biguanide—with most but not all infections quickly resolving. Failure can still occur occasionally and further research is needed on more effective combination chemotherapy. A number of guanidines have been identified in this paper that could be usefully pursued as part of combination chemotherapy along with the alkylphosphocholines.

Eye (2003) **17,** 893–905. doi:10.1038/sj.eye.6700563

Keywords: molecular epidemiology; drugs; *Acanthamoeba*; keratitis; incidence

Incidence

Three recent prospective cohort studies have investigated the incidence of microbial keratitis including that due to *Acanthamoeba* in defined populations. These cohort studies took place in the West of Scotland in 1995[1] when an annualised incidence rate was given of one case of *Acanthamoeba* keratitis in 6710 hydrogel contact lens wearers (CLW) (or 1.49 per 10 000), in Holland in 1996[2] with one case in 200 000 hydrogel CLW (or 0.05 per 10 000) and in Hong Kong in 1997–98[3] with one case in 33 000 hydrogel CLW (or 0.33 per 10 000). Only one patient was identified with *Acanthamoeba* keratitis in Holland, so the incidence figure is unreliable. Typical clinical features of *Acanthamoeba* keratitis are described in Seal *et al*.[4]

Three recent multicentre Questionnaire Reporting Surveys of *Acanthamoeba* keratitis took place in England within the last 10 years. The first in 1992–96 gave an incidence of one case in 39 370 CLW (or 0.25 per 10 000).[5] The second and third surveys in England and Wales took place in 1997–99 with one case in 47 620 CLW (or 0.21 per 10 000) and in 1998–99 with one case in 55 555 CLW (or 0.18 per 10 000);[6] 88% of CLW used hydrogel lenses and 12% used rigid lenses.

The latter two estimations for 1997–98 and 1998–99[6] have been corrected recently by Seal *et al*[7] to one case in 32 260 (or 0.31 per 10 000) and one case in 37 040 (or 0.27 per 10 000),

Applied Vision Research Centre, City University London EC1, UK

Correspondence:
DV Seal
Applied Vision Research Centre, City University London EC1, UK
Tel/Fax: +44 207 359 1475
E-mail: eyehealth@compuserve.com

Received: 28 February 2003
Accepted in revised form: 28 February 2003

Presented at Cambridge Ophthalmological Symposium on The Cornea 2002

respectively, on the basis that the incidence had been underestimated by approximately 33%. This occurred because their monthly Questionnaire Reporting Survey yielded an average return of only 67%. A high nonresponse rate over 30% is considered unreliable for making inferences to the whole population.[8]

However, it does appear that the incidence figure gained from the Hong Kong cohort study (0.33 per 10 000 CLW), where disinfection practice is similar to the UK with use of multipurpose cleaning and disinfecting solutions and use of one-step hydrogen peroxide products,[9] is remarkably similar to that of the corrected figures from the UK Questionnaire Reporting Survey (0.31 and 0.27 per 10 000 CLW). There are no reliable figures from the USA from either a cohort study or a reporting survey for comparison. This means that we can now expect approximately one case of *Acanthamoeba* keratitis per 30 000 hydrogel CLW per year (or 0.33 per 10 000) in England, Europe, Hong Kong, and other countries practising similar contact lens fitting and hygiene. The previous high rate in Scotland[1,10] can be attributed to the use of lens disinfection with chlorine tablets dissolved in tap water — a practice that has now been discontinued and has never been used in Europe, Hong Kong, or the USA.

The incidence of *Acanthamoeba* keratitis with gas-permeable and rigid contact lenses (CL) is much lower than with soft hydrogel CL and, consequently, no cases of the former were recorded in the three prospective cohort surveys in Scotland, Holland, and Hong Kong. There were 11 reports of rigid lenses being associated with *Acanthamoeba* keratitis (12% overall of CLW) in the combined England and Wales Questionnaire Reporting Surveys of 1997–98 and 1998–99.[6] Such cases have also been recognised in the past in the USA which implies that a very large population needs to be investigated to identify them. The estimated annualised incidence for *Acanthamoeba* keratitis in England and Wales for rigid lens wearers in 1997–99 then becomes one case per 285 715 (or 0.035 per 10 000), which has been corrected as described above for a 67% response rate in the Questionnaire Reporting Survey. This figure is 9.5 times lower than the expected incidence figure for soft or hydrogel lens wearers.

Recent studies from Korea[11,12] have found that *Acanthamoeba* can be isolated from 15% of contact lens storage cases. There are no incidence figures given for *Acanthamoeba* keratitis in the CLW population, but although the rate is reported as 'low', 12 clinical isolates were available for study. Riboprinting (see below) and mitochondrial DNA restriction fragment length polymorphism (RFLP) were used to show that storage case isolates had similar genetic characterisation to keratitis-causing strains and are thus potential pathogens for the cornea. The authors were able to analyse 43 isolates of *Acanthamoeba* from lens storage cases, which far exceeds the numbers isolated in Scotland, where the contamination rate was found to be approximately 2%[10,13] at the time of our highest incidence figures of *Acanthamoeba* keratitis in 1994/95. In Hong Kong, *Acanthamoeba* spp were isolated from the used lens storage cases of three out of 116 (2.6%) asymptomatic CLW control volunteers.[9] It is not clear why there is such a high isolation rate of *Acanthamoeba* from lens storage cases of students in Korea. It is suspected that tap water is used in contact lens storage case hygiene with inadequate disinfection.

Molecular epidemiology

The demonstrated human and animal pathogenicity of the genus *Acanthamoeba*[14,15] coupled with the difficulty of using morphological criteria for subgenus identification of isolates[16,17] has stimulated a number of laboratories to pursue molecular methods for the detection and identification of *Acanthamoeba*. The objectives are to discover a method, or methods, for detection and identification that are reliable, sensitive, relatively simple, and economical. The most reliable method would be based on the greatest amount of information about interstrain variation within the genus. At present, sequencing of nearly complete 18S rRNA genes satisfies this criterion more completely than any other method that has been tried. The reliability is based both on the number of variable sites within 18S rDNA and the number of strains for which sequences are available.[18] A recent analysis of complete DNA sequences of mitochondrial 16S rRNA genes from 68 *Acanthamoeba* strains fully supports the validity of the 12 genotypes previously identified using the nuclear 18S rRNA gene sequences.[19] Phylogenetic analyses of relatedness among isolates based on these sequences can be used as standards for evaluating measures of relatedness obtained using other methods.

Approaches that have been useful include RFLP analysis of the entire mitochondrial genome,[12,20–25] riboprinting (described below) of 18S rDNA,[12,26] production of type-specific PCR amplimers,[27–30] analyses based on sequences of subgenic PCR amplimers[30–32] as well as the development of fluorescent oligonucleotide probes for *in situ* staining that are specific either for the genus *Acanthamoeba* or for genotype T4.[33] The recent introduction of a 'reverse dot-blot' technique holds promise for the eventual simultaneous detection and identification of all rDNA genotypes present in any specimen.[34]

For many purposes, it is sufficient to identify amoebae at the genus level. In the case of *Acanthamoeba*, it has been difficult to classify isolates at the subgenus level even

when that would be useful.[16,17] Although taxonomic experts distinguish a number of species, inexperienced investigators have had a difficult time recognising them.

The subgenus classification used by us[30] is based on interstrain variation in 18S rDNA sequences. The 12 different rDNA genotypes (T1–12) identified are based on sequences from 65 *Acanthamoeba* isolates.[18,30] A 13th 18S rDNA genotype, designated ribotype T14, has recently been described.[34] The genotypes subdivide the three recognized morphological groups. A single genotype can consist, however, of multiple mitochondrial DNA RFLP groups and multiple species according to other methods of classification. For example, Lehmann *et al*[29] examined the ability of the ACARNA 1383:1655 primer set of Vodkin *et al*[27] to amplify a product from strains representing nine different mitochondrial DNA RFLP groups. Although the authors were careful to include a number of different species, probably only three of the 18S rDNA genotypes, T2 (*A. palestinensis*), T3 (*A. griffini*), and T4 (*A. castellanii* complex), were represented.

Rapid advances in the accuracy and rapidity of automated DNA sequencing technology, and the increasing application of automated sequencing facilities around the world, makes the use of PCR and DNA sequencing for identification of microbial isolates increasingly available. In our experience, the ability to culture *Acanthamoeba* from specimens is the most successful assay for these organisms. However, production of a colony can require a week or more. In addition, attention must be paid to varying nutrient and environmental requirements. For example, if the amoebae are encysted, excystment may require prolonged incubation for 4 weeks with heat-killed bacteria on agar plates sealed in plastic containers, to keep the agar moist, or use of a liquid medium such as peptone yeast-extract glucose (PYG) in tissue culture flasks. Schuster[35] has recently reviewed culture methods for various amoebae very well. Clinical isolates can be temperature-sensitive from prior drug treatment to the cornea and may not grow above 32°C.[36,37]

Thus, a sensitive method such as PCR with primers JDP1 and JDP2,[32] which is able to detect amoebae from all *Acanthamoeba* genotypes T1–12 in 1–2 days without the need for cell multiplication, is especially useful for clinical applications. It should be noted however that, although either version works, the primer JDP2 is correctly given in Booton *et al*,[32] whereas the sequence given in Schroeder *et al*[30] is incorrectly missing one internal G. Success in detection can be greater than 90% if the PCR and sequencing are used in combination with a genus-specific fluorescent *in situ* hybridisation (FISH) T4 probe, another approach that can be completed in 1–2 days.[33]

Although it is believed that sequencing of 18S rDNA is the most reliable indicator of strain relationships at this time, riboprinting can be used instead to produce similar dendrograms. Riboprinting involves PCR followed by RFLP analysis of complete 18S rRNA genes.[12] Although comparisons using all the same strains are unavailable, phylogenetic trees based on RFLP riboprints[12,26] appear to identify clusters of related strains similar to those identified based on DNA sequencing.[18] The use of mitochondrial 16S rDNA for riboprinting has the advantage that it avoids distortions of interstrain relationships caused by introns that occasionally occur in the nuclear 18S rRNA genes of some strains. The use of DNA sequences for interstrain comparisons, however, provides more information about sequence similarities and differences and can be limited to homologous regions of the DNA.

Classification of *Acanthamoeba* based either on DNA sequence types (genotypes) or RFLP riboprints provides consistent measures of evolutionary relatedness. Sequencing of short diagnostic fragments of 18S rDNA also can be used effectively to differentiate individual strains within genotypes.[32] Another recent study demonstrates advantages of using mitochondrial 16S rDNA sequences for identifying subgenotype clusters.[19] Correlations of these molecular characters with species names is problematical, however, because they show that *Acanthamoeba* strains with the same species name sometimes are less closely related than strains with different species names. Thus, correlations always should be made relative to species type-strains when they are available.

These techniques can be used for epidemiological tracking of *Acanthamoeba* to identify sources of infection with more certainty which then allows the development of better prevention. The approach using RFLP of whole-cell DNA was first used by Kilvington *et al*[22] in 1991 to investigate the relationship of strains cultured from infected corneas of CLW and from the environment, following the suggestion that infection may arise from contamination of contact lenses with domestic tap water.[38,39] Gautom *et al*[23] conducted a similar study.

A much more refined technique, PCR of 18S rDNA with complete sequencing of 2800 base pairs, was used to compare isolates from the cornea, lens storage case, and domestic tap water which were shown to be identical and to have homology with the gene sequence of *A. griffini* type strain which is also genotype T3.[40] In addition, there was a unique group I intron located within the small subunit rDNA which had identical sequences for the three isolates. This implied beyond doubt in this patient that domestic tap water was the source of the corneal infection.

More recent work, however, has shown that the situation described above does not always occur and that the epidemiology can be more complicated. Analysis of *Acanthamoeba* isolates from a cohort study of microbial keratitis in Hong Kong,[3] using 18S rDNA typing and subtyping, has found that isolates in the home water supply can be different from those infecting the cornea.[32] Identification of *Acanthamoeba Rns* genotypes was based on sequences of approximately 113 base pairs within the genus-specific amplicon ASA.S1.[30] Of 13 isolates from corneal scrapes, lenses, or lens storage cases, 12 were *Rns* genotype T4 (the commonest genotype associated with keratitis) and the remaining one from a noncorneal source was T3. From one patient, the same isolate was found from the corneal scrape as from the contact lens (T4/6) while different types were found in the kitchen tap water (T3/2) and bathroom basin drain (T3/3). Similarly, in another patient, type T4/6 was isolated from the contact lens but type T3/4 from the basin tap water. Overall, all isolates from the cornea were type T4 but those from the domestic tap water were either T3 (occasional cause of keratitis) or type T4 (pathogenic to the cornea) in 50–50 proportions. Use of subtyping in this way has identified that the source of *Acanthamoeba* infection need not be from the domestic tap water. The question then becomes 'where has the infection originated from?'. Investigators should consider other water sources including those at work, previously reported as a likely source of infection in a patient 'MT' by Hay *et al*,[37] and sites where the CLW may wash their lenses outside the home or expose them to water such as swimming pools, showers, jacuzzis, fountains, water sports, etc.

Recent epidemiological findings include isolations of *Acanthamoeba* of genotype T4 from sandy beaches,[41] which may be relevant to those wearing extended-wear silicone-hydrogel contact lenses which are particularly 'sticky' for this amoeba.[42]

Acanthamoeba has been isolated before from seawater, ocean sediment, chlorinated swimming pools (which can contain up to 10^4 amoebae per litre as they are resistant to chlorine) and a variety of other types of water[43] as well as from sewage outfalls.[30] In addition, a relationship between the presence of *Acanthamoeba* and faecal indicator bacteria has been found in samples of ocean sediment.[44] Interestingly, isolates of *Acanthamoeba* genotype T4, associated with keratitis in humans, have been cultured recently from the organs of freshwater fish.[31] *A. griffini*,[45] *A. culbertsoni*,[46] and *A. royreba*[47] have also been isolated from fish but their genotype, while not performed on these particular isolates, is likely to be T3 (occasional cause of keratitis), probably T10 (nonkeratitis) and T4 if the isolate was similar to *A. royreba* (Oak Ridge type-strain), respectively. Chung *et al*[26] identified by riboprinting the 'Chang' strain of *A. castellanii* (T4 equivalent) from fresh water and found it closely related to *A. hatchetti* (T11 equivalent) isolated from ocean sediments (salt water); both strains were expected to be pathogenic in animal models. The relationship between *Acanthamoeba* genotype T4 causing keratitis in humans and T4 isolates from organs of fishes, where its role in surviving without causing disease is that of a protozoon parasite, is intriguing.

Genotyping, based on the scheme developed by Stothard *et al*,[18] has been used by Walochnik *et al*,[48–50] and Khan *et al*[51] to identify types of *Acanthamoeba* causing keratitis and to pursue correlations with pathogenicity. Walochnik *et al*[50] have identified a keratitis-causing strain belonging to type T6. This isolate was morphologically identical to T4 strains, which belong to group II of Pussard and Pons.[16] However, the only other type T6 isolate described, associated with *A. palestinensis* 2802,[18] belongs to morphological group III. Interestingly, their isolate of type T6 was shown to be immunologically unique from genotype T4.[50]

Khan *et al*[51] studied six 'pathogenic' isolates from cases of keratitis of which five were type T4 and one was type T11, the first such report. Type T11 had been previously recognised by Walochnik *et al*[48] colonising a contact lens storage case of a noninfected individual. Type T11 is closely related to T4 species.

Walochnik *et al*[50] have concluded that the results of their studies support the *Acanthamoeba* 18S rDNA sequence type classification of Stothard *et al*.[18] In addition, Khan *et al*[51] have used the same scheme to distinguish pathogenic-associated types T4, T3, and T11 from nonpathogenic (for the eye) associated types T2, T7, and T9. Each genotype however has heterogeneity and can be further investigated by either subgenotype sequencing, 18S rDNA PCR-RFLP (riboprinting), or mitochondrial DNA-RFLP. This approach is considered to be of more value to taxonomists and epidemiologists than riboprinting as a primary investigative tool, with attempts to relate patterns gained to those of morphologically based species—an inexact science at best.

New drugs for treatment

The first effective treatment of *Acanthamoeba* keratitis was developed 20 years ago with propamidine (Brolene) and neomycin[52] but only half the patients responded. After considerable research, this regime was replaced 7 years ago with chlorhexidine (bis-biguanide) and propamidine[37,53] or the polymeric equivalent 'polyhexamethylene biguanide' (PHMB). PHMB was originally combined with propamidine[54,55] but is now combined with hexamidine (Desmodine) (J Dart, 2002, personal communication).

The use of the biguanides has revolutionised the treatment of early cases of *Acanthamoeba* keratitis which respond within 1 week[53] and has been very successful for cases presenting within 8 weeks. Relief of pain is rapid and the active infection is usually eradicated within 4 weeks as opposed to 4 months or many more with propamidine and neomycin. However, late-presenting cases after the infection has been established for 3 months or more remain particularly difficult to treat requiring prolonged drug therapy as well as graft surgery.[4]

There is a remaining conundrum in some late-presenting cases with deep stromal infection. *Acanthamoeba* can persist causing an active infection despite treatment with chlorhexidine or PHMB but, when isolated and cultured, are found to be sensitive to these compounds. It is not understood why the biguanides can be ineffective *in vivo*. Further drugs are needed in this situation and study of additional guanidino group compounds could be valuable.

The guanidino 'family' is given in Table 1 with a list of drugs or chemicals containing guanidino groups that have been investigated in Glasgow for their effect on *Acanthamoeba*.

All the drugs listed in Table 1 were investigated for their effect on 20 clinical isolates of *Acanthamoeba* cultured from cases of keratitis (14 from Moorfields Eye Hospital, London and six from Western Infirmary, Glasgow). Methods used in our laboratory were similar to those reported before by Hay *et al*.[37] Essentially, double dilutions of each drug were performed in microtitre plates with the trophozoites and cysts at a concentration of 2×10^4 organisms per 100 µl of medium per well. Microtitre plates were incubated at 32°C for 48 h. Sensitivity of isolates was determined microscopically by recording the lowest concentration that gave complete lysis or degeneration of trophozoites (minimum trophozoite amoebicidal concentration or MTAC). For cysts, the lowest concentration that resulted in no excystment was recorded (minimum cysticidal concentration or MCC), after washing the cysts free of residual drug and reincubation in a defined fluid medium[56] for 7 days.

The modal values for the MTAC and MCC and the range (in µg/ml) for each drug, when tested against the 20 clinical isolates, are given in Figures 1a–f. A number of guanidino-containing compounds were unexpectedly effective against trophozoites including those with an MTAC at <10 µg/ml (alexidine, PHMB, chlorhexidine, hexamidine, propamidine, phenformin, ismelin solution, and dicyanodiamine), at 10–20 µg/ml (pentamidine, diminazene, and dibromopropamidine), and at 20–30 µg/ml (Lapudrine, Paludrine, Proguanil, moroxydine, metformin, ofloxacin, and fumigillin). Those with MCCs that were similarly effective against cysts as trophozoites only included the bis/polymeric biguanides of chlorhexidine and PHMB. MCCs that were higher than MTACs included alexidine ($\times 5$), the diamidines ($\times 2$ to $\times 4$), Paludrine ($\times 2.5$), phenformin ($\times 5$), ismelin solution ($\times 5$), and fumigillin ($\times 5$). Other compounds had MCCs of >100 µg/ml and would be ineffective for clinical treatment because of toxicity at high concentrations.

For drugs to be effective against cysts they must penetrate the cyst wall and act on the internalised amoeba. It is believed, but not proven, that chlorhexidine and PHMB act by binding of their highly charged positive molecules to the mucopolysaccharide plug of the ostiole, resulting in penetration through it to the internalised amoeba, where they bind to the

Table 1 'Guanadino' family of drugs investigated for their effect on 20 clinical isolates of *Acanthamoeba* cultured from cases of keratitis

Amino acids
L-arginine and D-arginine (C=NH, -NH_2)
Steric biguanides
Bis-biguanide (chlorhexidine), alexidine, polyhexamethylene biguanide (PHMB)
Diamidines
Propamidine (Brolene), dibromopropamidine (Golden Eye Ointment), methylglyoxal bis(guanylhydrazone)[a] (MGPG), hexamidine (Desmodine), pentamidine (Pentacarinat), diminazene (Berenil)
Antimalarial guanides
Chlorguanide (Proguanil), chlorguanide HCl (Paludrine), chlorproguanil (Lapudrine)
Nonsteric guanides
Abitilguanide HCl (Moroxydine), phenethyl diguanide HCl (Phenformin), 1,1 dimethyl biguanide HCl (Metformin)
Guanidino derivatives
4 aminobutyl guanidine (Agmatine), cyanoguanidine (Dicyanodiamide), aminoguanidine bicarbonate, guanidino acetic acid, guanethidine monosulphate (Ismelin tablet and solution)
Other guanidino-containing compounds and fumigillin
Famotidine (Pepcid), phenylene diamine, 4-OH quinolone derivative (Ofloxacin), fumigillin

[a]Not tested here.

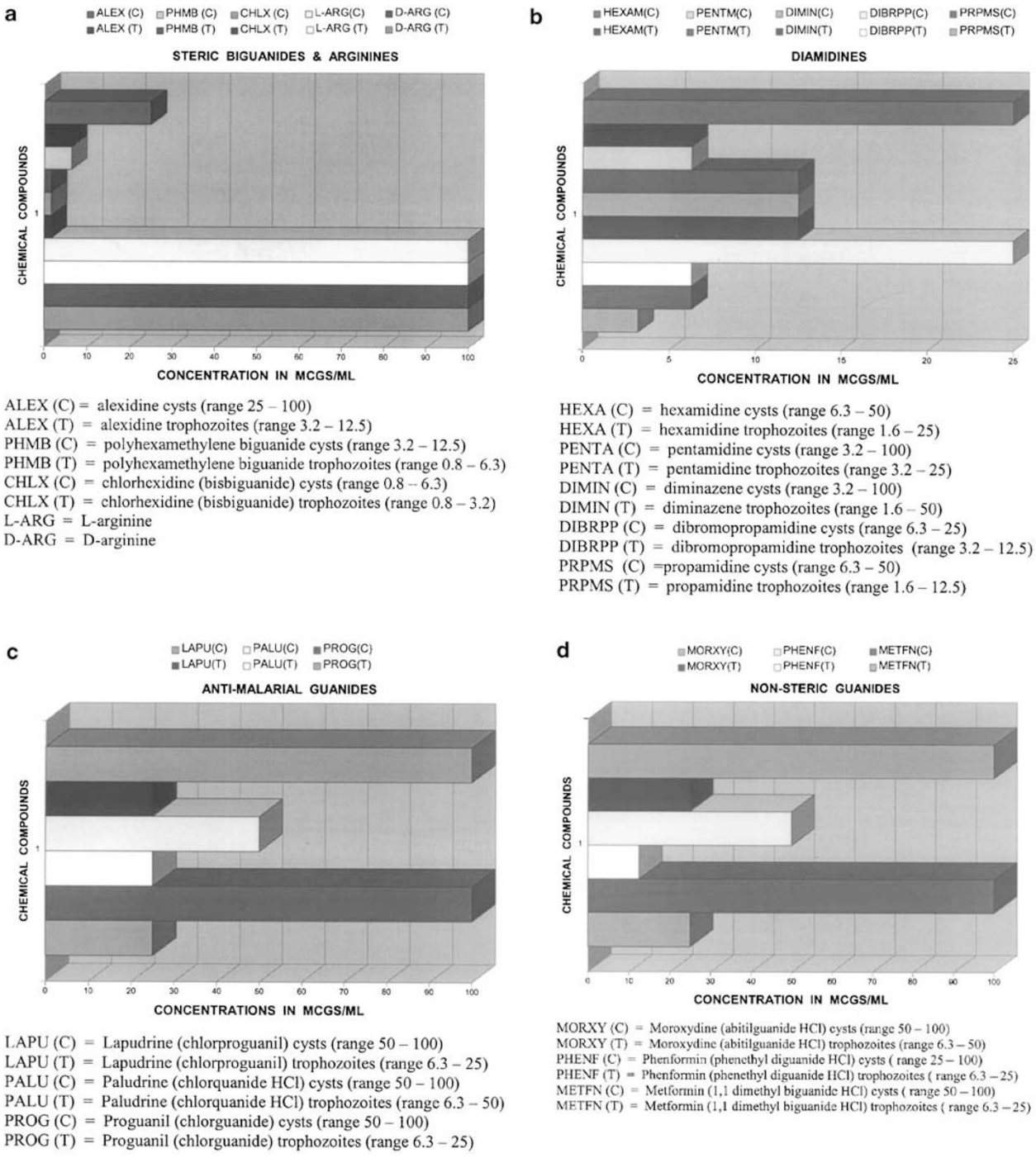
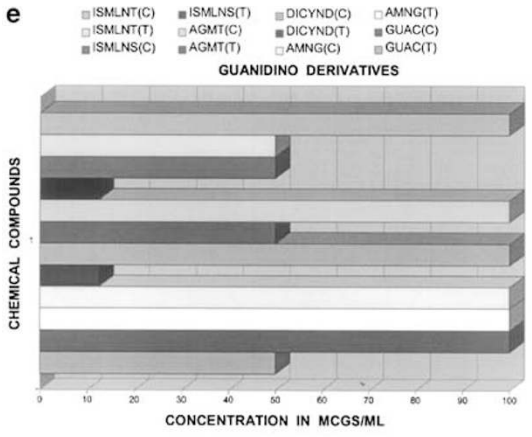


Figure 1 Modal values (μg/ml) for cysts and trophozoites.

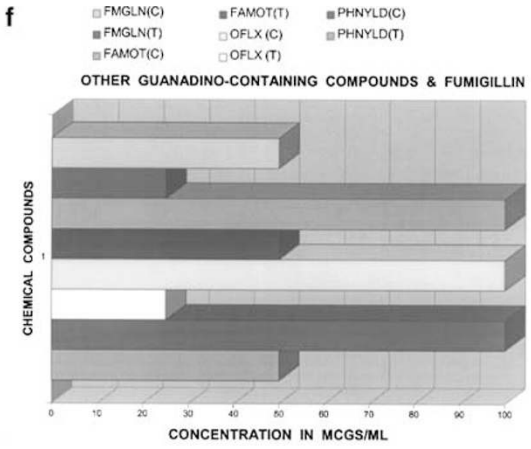
phospholipid bilayer of the cell membrane of the internalised amoeba. This results in membrane damage with irreversible loss of calcium firstly, and then cell electrolytes, from the cytoplasm to cause cell lysis and death. The diamidines also have a membrane damaging action.

Steric biguanides

MTACs and MCCs in μg/ml for chlorhexidine and PHMB were performed by Elder *et al*[57] when mean values (range) gained were MTAC 0.71 (0.49–1.9) and 0.6 (0.49–0.97) and MCC 2.77 (0.49–15.6) and 2.2 (0.49–3.9),



ISMLNT (C) = Ismelin (guanethidine) tablet cysts (range 50 – 100)
ISMLNT (T) = Ismelin (guanethidine) tablet trophpozoites (range 25– 50)
ISMLNS (C) = Ismelin (guanethidine) solution cysts (range 25 – 50)
ISMLNS (T) = Ismelin (guanethidine) solution trophozoites (range 6.3 – 25)
AGMT (C) = Agmatine (4aminobutylguanidine) cysts (range 100)
AGMT (T) = Agmatine (4aminobutylguanidine) trophozoites (range 50 - 100)
DICYND (C) = Dicyanodiamide (cyanoguanidine) cysts (range 25 – 100)
DICYND (T) = Dicyanodiamide (cyanoguanidine) trophozoites (range 3.2 – 25)
AMNG (C) = aminoguanidine bicarbonate cysts (range 100)
AMNG (T) = aminoguanidine bicarbonate trophozoites (range 100)
GUAC (C) = guanidino acetic acid cysts (range 50 – 100)
GUAC (T) = guanidino acetic acid trophozoites (range 25 – 50)



FMGLN (C) = Fumigillin cysts (range 25 – 100)
FMGLN (T) = Fumigillin trophozoites (range 6.3 – 50)
FAMOT (C) = famotidine (Pepcid) cysts (range 50 – 100)
FAMOT (T) = famotidine (Pepcid) trophozoites (range 25 – 50)
OFLX (C) = Ofloxacin (4OH quinolone derivative) cysts (range 50 – 100)
OFLX (T) = Ofloxacin (4OH quinolone derivative) trophozoites (range 12.5 - 25)
PHNYLD (C) = phenylene diamine cysts (range 50 – 100)
PHNYLD (T) = phenylene diamine trophozoites (range 25 – 100)

Figure 1 (*Continued*).

respectively. The results from our work differ slightly in finding the MCC to be lower for chlorhexidine than for PHMB. There is no advantage to be gained by treating with PHMB and chlorhexidine — one or the other should be used. The combination will be more likely to be toxic.

Burger *et al*[58] investigated killing kinetics of PHMB for *Acanthamoeba*. They found that fewer than 2% of cysts in PYG medium survived 30 s of exposure at a concentration of 45 ppm (or μg/ml). Further exposures of 15 min and 1 h killed greater than 90% of those cysts surviving initial killing.

Khunkitti *et al*[59] investigated the lethal effects of biguanides on trophozoites and cysts of *A. castellanii* using amoebae derived by axenic culture. Washed cells were added to the biguanides at 10^5 amoebae/ml and held at 20°C for 4 h only. Samples were then neutralised, spun, and cultured on agar plates with live *E. coli* so that growth of *Acanthamoeba* gave plaques. Lethal effects were studied with differing contact times and formulations. The authors found that chlorhexidine and PHMB each had an MCC of 25 μg/ml. However, while the MCC for chlorhexidine was reduced in 0.1% w/v EDTA to 12.5 μg/ml, that for PHMB increased to 100 μg/ml. For 0.1% w/v EDTA-Tris buffer at pH 7.8, the MCC for chlorhexidine was 3.13 and that for PHMB was 25, while for 0.1% w/v EDTA-borate buffer the values were 12.5 and 50, respectively. This is of concern as multipurpose solutions for disinfecting contact lenses contain PHMB at 1 μg/ml (= 1 ppm or 0.0001%) often with EDTA as buffer which will thus increase or decrease its activity according to the formulation.

Narasimhan *et al*[60] compared the effect of PHMB with chlorhexidine on cysts, cultured at 10^4/ml on agar, derived from 19 clinical isolates of *Acanthamoeba* keratitis. These cysts were exposed to the chemicals for 48 h in glass Durham tubes. Cysts were washed free by centrifugation and then cultured on non-nutrient agar seeded with *E. coli* and incubated at 37°C for 7 days. Any growth represented a failure of a 4 $\log_{10}$ kill effect. The mean MCC value (µg/ml) for PHMB was 55 (range 25–100) and for chlorhexidine was 33 (range 1.5–100), with an MCC_{50} of 50 and 25, respectively. Anti-*Acanthamoeba* activity of chlorhexidine was greater than that of PHMB ($P = 0.036$). The higher values gained in µg/ml than Elder *et al*,[57] Hay *et al*,[37] and Seal *et al*[53] are due to the method used; a single surviving cell will have been cultured and interpreted as a failure. In practice, a 5 $\log_{10}$ kill effect is needed to inactivate totally an inoculum of 10^4 trophozoites or cysts. The concentration used in clinical practice of 0.02% eye drops (200 µg/ml) should be maintained. Increasing the concentration especially of chlorhexidine is likely to be toxic to the eye for reasons given in Seal *et al*.[53]

Diamidines

Perrine *et al*[61] investigated the amoebicidal effect of various diamidines against *A. polyphaga* measuring the

Table 2 Comparative effectiveness (μg/ml) of the diamidines and steric biguanides against 20 clinical isolates of *Acanthamoeba* and reported values by others

	MTAC (μg/ml) Modal value	*MTAC (μg/ml) Range*	*MCC (μg/ml) Modal value*	*MCC (μg/ml) Range*
Propamidine	3.2	1.6–2.5	6.3	6.3–50
Propamidine[57]	0.6 (mean)	0.5–1.0	46.0 (mean)	2–500
Dibromopropamidine	6.3	1.6–12.5	25.0	6.3–25
Hexamidine	6.3	1.6–25.0	25.0	6.3–50
Pentamidine	6.3	6.3–12.5	6.3	3.2–100
Pentamidine[57]	0.6 (mean)	0.5–1.0	81.2 (mean)	1–500
Diminazene	12.5	3.2–25.0	12.5	3.2–100
Chlorhexidine	1.6	0.8–3.2	1.6	0.8–6.3
PHMB	1.6	0.8–6.3	6.3	3.2–12.5

MTAC: minimum trophozoite amoebicidal concentration; MCC: minimum cysticidal concentration.

survival times rather than the MTAC and MCC. This method allows the diamidines to be compared within their study but not with other studies which is unfortunate. They investigated the effect of increasing the alkyl chain length $(CH_2)n$ connecting the two benzene rings in the molecule. They found an increased amoebicidal effect for both trophozoites and cysts from propamidine ($n=3$) to hexamidine ($n=6$) to nonamidine ($n=9$), when there is an increase in the lipophilic property of the molecule.

They also investigated lethal effect kinetics which they found unchanged by the elongation of the alkyl chain and concluded that the amoebicidal activity arose from the cationic surface-active properties induced by the protonated amidine group attached to the benzene rings. This is likely to be concentration dependant however and it would be surprising if DNA intercalation was also not involved as well as an effect on polyamine metabolism (see below).

The findings of Perrine *et al*[61] support the replacement of propamidine (Brolene) by hexamidine (Desmodine) available commercially in France as 0.1% eye drops. These observations are supported by Brasseur *et al*[62] who carried out 100% killing tests on isolates from two patients, comparing propamidine and hexamidine. They found that trophozoites required 9 and 4 or 7 and 5 h, and cysts 6 and 3 or 7 and 3 days, respectively, for kill by propamidine or hexamidine. The clinical effectiveness of monotherapy with hexamidine was limited and prolonged and far less efficient than with biguanides.

The results given in this paper for the MTAC and MCC tests, using the commercial preparation of hexamidine (Desmodine), disagree with those of Perrine *et al*[61] and Brasseur *et al*[62] and find it to be less effective than propamidine. These and other results are given in Table 2. All the diamidines were more effective against trophozoites than cysts by a factor of $\times 2$–$\times 4$. Pentamidine (Pentacarinat) and diminazene (Berenil, licensed for veterinary use) are used systemically for the treatment of pneumocystis in humans and trypanosomiasis in cattle respectively and could theoretically be used for topical treatment of *Acanthamoeba* keratitis but this has not yet been reported. Careful use topically with these two diamidines would be appropriate in extraneous circumstances if other drugs were not available.

Treatment of *Acanthamoeba* keratitis 14 years ago with monotherapy of propamidine followed by combination with arsenic (as an atoxyl derivative), in the absence of neomycin due to hypersensitivity, led to the development of a fulminant infection with a propamidine and arsenical resistant isolate[36] (Figures 2a and b and Table 3). Similar arsenical drugs were investigated by Jennings[63] for their effect on a mouse model of African trypanosomiasis. It was shown that arsenical compounds combined with difluoromethylornithine (DFMO, Eflornithine) in a critical way led to a rapid cure. This led to the suggestion that the trypanothione oxidation–reduction system of the trypanosome was the main target of the drug combination. This was later developed into a combination of the arsenic as melarsoprol (Arsobal, Mel B) and a 5-nitroimidazole drug (Megazol) when a single application was curative for the mouse model.[64,65] Resistance is developing however in field conditions owing to both arsenicals as melarsoprol and to the diamidine diminazene (Berenil);[66] drug resistance has been recognised as a result of altered novel transporters, specific targets, or activation of the drug which suggests that mutation has occurred.

DFMO (Eflornithine) was investigated by Hay *et al*[37] and found to have no effect against *Acanthamoeba* either alone or synergistically in combination with arsenic as cymelarsan. Cymelarsan gave a weak effect against trophozoites (MTAC 40 μg/ml) with no effect on cysts (MCC > 100 μg/ml).

Resistance to propamidine in our patient's isolate of *Acanthamoeba* was thought due to mutation as it was

associated with temperature sensitivity when resistant isolates failed to grow at >32°C.[36,37] 18S rRNA gene analysis identified three alleles in each of the sensitive and resistant isolates confirming previous work showing that they had identical mitochondrial RFLPs.[22,67] The combination of both techniques is highly supportive of the propamidine resistance and temperature sensitivity being due to mutation but we have not identified its location. The first (drug-sensitive) isolate was of the T4 genotype[18] and the resistant isolates probably were too because they have very similar sequences.

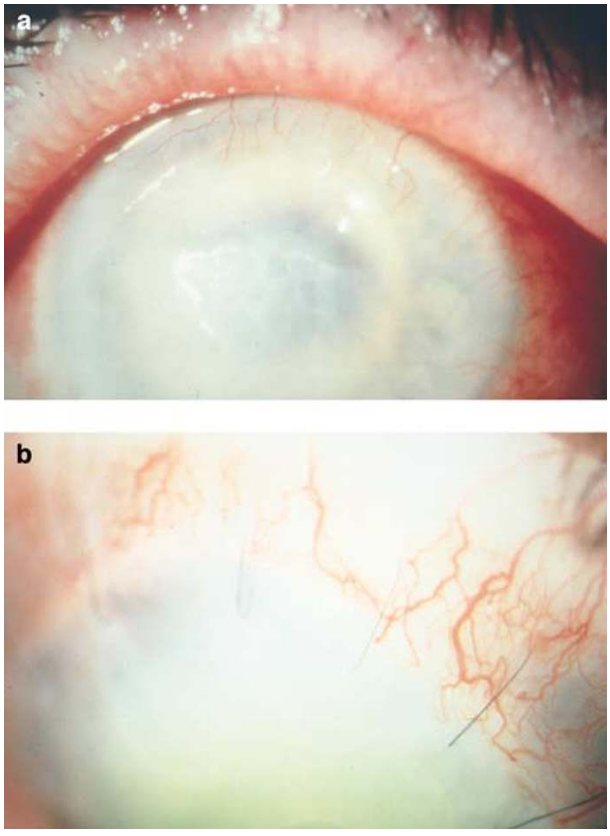


Figure 2 (a) Severe keratitis owing to progressive uncontrolled *Acanthamoeba* infection. (b) Severe recurrent keratitis following a corneal transplant owing to an arsenic and propamidine-resistant *Acanthamoeba* infection.

Table 3 gives the MTACs and MCCs to arsenic, the other diamidines, and steric biguanides for the propamidine-sensitive and -resistant isolates described above. It can be seen that there is cross-resistance with the other diamidines which provides further evidence for the resistance being likely due to mutation. Arsenic resistance also developed. Interestingly, the propamidine-sensitive and -resistant isolates were both sensitive to chlorhexidine and PHMB.

Monotherapy has been used in France with Desmodine without problems.[62] In the UK, many optometrists and pharmacists issue propamidine (Brolene) as an over-the-counter eye drop medicine for sore, red eyes not needing a prescription, with over 3 million doses issued per year. A few of these red eyes in CLW are likely due to early *Acanthamoeba* infection. No other reports have been made of progressive or resistant infection associated with this monotherapy. It seems probable therefore that the combination of propamidine with arsenic was the reason that mutation occurred giving resistance to both drugs. It is probably wise not to introduce arsenicals into the treatment of *Acanthamoeba* keratitis.

Diamidines, which are also guanidino-group compounds, exert part of their activity by intercalating with DNA by binding to the minor groove of A/T-rich domains.[68] The relation between this nucleic acid binding

Table 3 Comparative effectiveness of arsenic, various diamidines, and biguanides against a temporal sequence of isolates that became temperature-sensitive mutants[36,37,67]

	Modal		*MTAC and MCC (μg/ml)*			
	February (on propamidine and arsenic)		*March*[a,b]		*July*[a,b]	
	Trophs	*Cysts*	*Trophs*	*Cysts*	*Trophs*	*Cysts*
Arsenic (1989)	2.5	10.0	>150	ND	>150	ND
Propamidine(1989)	1.6	3.2	50.0	200.0	50.0	200.0
Propamidine	6.3	6.3	12.5	25.0	ND	ND
Dibromopropamidine	12.5	6.3	12.5	25.0	6.3	25.0
Hexamidine	6.3	12.5	12.5	50.0	12.5	25.0
Pentamidine	6.3	6.3	6.3	50.0	25.0	100.0
Diminazene	1.6	3.2	3.2	3.2	6.3	25.0
Chlorhexidine	0.8	1.6	1.6	1.6	0.8	0.8
PHMB	1.6	3.2	1.6	3.2	1.6	6.3

[a]Temperature-sensitive mutants with growth at <32°C.[36]
[b]Therapy with paromomycin, benzethonium chloride, clotrimazole, and a phenanthridinium compound (R11/29).
ND: not done; MTAC: minimum trophozoite amoebicidal concentration; MCC: minimum cysticidal concentration.

and that by the antineoplastic guanidino-group compounds is not known but it may be relevant. There could be similarity with the APCs, albeit nonguanidino-containing compounds, which have both antineoplastic activity on human cells and are effective against protozoan parasites. Diamidines interefere with polyamine metabolism which is reviewed below.

The polyamine metabolism in the protozoan or human cell can be summarised as follows:

Arginine— ————>Ornithine————*————
>Putrescine————**————>Spermidine
*ornithine decarboxylase (ODC),
**decarboxylated *S*-adenosylmethionine (SAMDC)

Gupta *et al*[69] reported that the diamidine MGBG (see cancer chemotherapy section) enhanced starvation-induced encystment by 90% through its action as an inhibitor of decarboxylated *S*-adenosylmethionine. Byers *et al*[70] showed that this action is weak and reversible. This is different from its effect on exponential phase cultures (see cancer chemotherapy section) when it acted as an inhibitor of ODC and the cultures remained as trophozoites. In contrast, propamidine, diminazene aceturate, and pentamidine are good inducers of encystment.[71] They are irreversible inhibitors of SAMDC in mammals, trypanosomes, acanthamoebae, and humans, and decrease polyamine levels in amoebic cells during encystment. The encystment can be blocked by the addition of putrescine or spermidine. Their other targets for inhibition are the mitochondria. Diamidines also have a membrane damaging effect similar to but less effective than the steric biguanides.

Cancer chemotherapy drugs containing guanidino groups

Inhibition of polyamine metabolism has been investigated for over 20 years as a means of producing an antitumour effect that might be applicable for therapy. Interestingly, this action also has an inhibitory effect on protozoan parasites. New data exist for guanidino-containing drugs in cancer chemotherapy that might be effective against *Acanthamoeba*.[72] *m*-Iodobenzylguanidine (MIBG) and the diamidine methylglyoxal bis(guanylhydrazone) 'MGBG' have established use as anticancer drugs. MIBG has structural similarity to norepinephrine. MGBG is a structural analogue of the natural polyamine 'spermidine'.

Byers *et al*[70] added MGBG to an exponential phase culture of *A. castellanii* (strain Neff) and found that it acted like an inhibitor of ornithine decarboxylase. They showed that multiplication was inhibited and that no encystment occurred. Multiplication was restored by the addition of extracellular polyamines. The question arises as to whether we have tricked the amoeba to think that it has increased levels of spermidine, and hence stays in the trophozoite form, or we have switched off polyamine metabolism, and with it the multiplication of the *Acanthamoeba*, so that it remains as a trophozoite.

CHS828 is a pyridyl cyanoguanidine newly recognised for both its cytotoxic and antihypertensive activities. These three drugs also inhibit mitochondrial function. Laboratory studies are required to test their efficacy against *Acanthamoeba* both separately and combined with either chlorhexidine/PHMB, for increased activity and penetration of any cyst via its ostioles, propamidine/hexamidine, for possible synergy involving both DNA intercalation and cell membrane damage, or with alkylphosphocholine-1 (see below).

Guanides and guanidino derivatives

The antimalarial compounds (Proguanil, Paludrine, and Lapudrine) have considerable effect against trophozoites with MTAC values as low as 10 μg/ml but are disappointing against cysts (MCC values >100 μg/ml). There is scope to combine these antimalarial compounds with other drugs such as chlorhexidine for enhanced activity for topical treatment or with pentamidine for systemic therapy of granulomatous amoebic meningoencephalitis or with the alkylphosphocholines (APCs) (see below).

An alternative strategy is to hold the amoeba in its trophozoite form by using diamidines selectively to interfere with polyamine metabolism. MGBG could be suitable for this purpose (see diamidine and cancer chemotherapy sections). However, MGBG also inhibits multiplication of the trophozoite which could reduce the activity of the antimalarial drug.

Other compounds with unexpected activity against trophozoites included the oral hypoglycaemic drugs phenformin and metformin (Figure 1d) and the quinolone antibacterial drug ofloxacin (Figure 1f). However, they all lacked activity against cysts (MCC values >100 μg/ml).

Alkylphosphocholines

A new nonguanidino group compound (alkylphosphocholine-1, APC-1) has been shown recently to have excellent trophozoiticidal activity effective against 10^5 trophozoites of *Acanthamoeba* but with only partial cysticidal activity (3 $\log_{10}$ reduction from an inoculum of 5 $\log_{10}$).[73] This group of compounds consists of phosphocholine esterified to long-chain aliphatic alcohols exhibiting *in vitro* and *in vivo* antineoplastic activities. In addition, they are active against protozoan

parasites such as *Leishmania donovani*, *Trypanosoma cruzi*, and *Entamoeba histolytica*. Combination with chlorhexidine or PHMB would be expected to give an enhanced cysticidal effect.

APC-1 (hexadecyl phosphocholine, 'Miltefosine') has been used orally to successfully treat visceral leishmaniasis.[74] Walochnik *et al*[73] found that APC-1, out of eight compounds with increasing chain length, had the highest level of amoebicidal activity similar to findings of others for *Leishmania* sp. and *T. cruzi*. Exposure to APC-1 results in vacoulisation, rounding up of cells, and lysis within 1 h. The mechanism of action of APC is not clear at present but, at high concentrations, the membrane architecture is the primary target. APC-1 cannot be given intravenously because of toxicity. Oral therapy causes side effects but they can be tolerated. Careful evaluation is needed for possible topical therapy of *Acanthamoeba* keratitis, being aware that alone it is not cysticidal.

Imidazoles

Elder *et al*[57] investigated the MTAC and MCC (μg/ml) for ketoconazole, fluconazole, miconazole, and clotrimazole. They found MTACs of 144 and 320 for ketoconazole and fluconazole and MCCs of >500 for all four compounds. These levels are much higher than those of Ficker *et al*,[36] who recorded MTACs of between 7 and 19 and MCCs between 7 and 75.

Imidazoles are applied as 1% eye drops (10 000 μg/ml) in arachis oil which should be effective against trophozoites but may not be for cysts. Drug levels achieved in the cornea by the topical route for itraconazole vary from 200 to 250 μg/g tissue according to the type of vehicle used including balanced salt solution, polyvinyl alcohol, boric acid, and olive oil.[75] Oral itraconazole penetrates into the cornea to treat fungal infection satisfactorily but levels of only 0.05 μg/g tissue are achieved[76] which are far too low to be even trophozoiticidal; for oral ketoconazole, a higher level of 0.5 g/g tissue has been recorded but this is still insufficient.

Imidazoles should never be used alone to treat *Acanthamoeba* infection but always in combination therapy and applied by the topical route.

Neomycin

This should no longer be used for the treatment of *Acanthamoeba* keratitis. The cysts are almost always resistant and treatment can result in the development of both neomycin-resistant and temperature-sensitive mutants that will grow well at 25°C but weakly at 35°C as described in a patient [AT].[37] In addition, neomycin can promote hypersensitivity to itself.

Anti-inflammatory drugs

Niederkorn *et al*[77] have demonstrated that dead cysts persist in the corneal stroma and remain antigenic. This can give rise to a serious inflammatory reaction. Clinicians should be careful to avoid use of corticosteroids if possible because they suppress the activity of the macrophage, which is the 'scavenger' phagocytic cell responsible for host immunity to *Acanthamoeba*. Use of nonsteroidal anti-inflammatory drugs, particularly flurbiprofen (Froben), is encouraged and also acts as an analgesic and mydriatic.

Acknowledgements

I am grateful to George Gettinby, Magnus Peterson, Crawford Revie, and Eric Wong for various discussions related to incidence and Questionnaire Reporting Surveys. I am grateful to my colleagues Elizabeth Houang, Dorothy Fan, and Dennis Lam for the opportunity to have worked with them in Hong Kong. I am also grateful to Tom Byers and Greg Booton for all their help with the molecular biology that has been undertaken with the *Acanthamoeba* isolates from the UK and Hong Kong. I also thank Ruth Connor and John Hay for their scientific assistance with drug testing of the isolates, Tara Beattie and Alan Tomlinson for good advice, and Philip Thomas for advice on imidazoles.

References

1 Seal DV, Kirkness CM, Bennett HGB, Peterson M, Keratitis Study Group. Population-based cohort study of microbial keratitis in Scotland: incidence and features. *Contact Lens Ant Eye* 1999; **22**: 49–57.
2 Cheng KH, Leung SL, Hoekman HW, Beekhuis Wh, Mulder PG, Geerards AJ *et al*. Incidence of contact lens associated microbial keratitis and its related morbidity. *Lancet* 1999; **354**: 181–185.
3 Lam D, Houang E, Lyon D, Fan D, Wong E, Seal D. Incidence and risk factors for microbial keratitis in Hong Kong: comparison with Europe and North America. *Eye* 2002; **16**: 608–618.
4 Seal DV, Bron AJ, Hay J. *Ocular Infection — Investigation and Treatment in Practice*. Martin Dunitz: London, 1998, pp 1–275.
5 Radford CF, Lehmann OJ, Dart JKG. *Acanthamoeba* keratitis: multi-centre survey in England 1992-1996. *Br J Ophthalmol* 1998; **82**: 1387–1392.
6 Radford CF, Dart J, Minaissian D. *Acanthamoeba* keratitis in England and Wales: incidence, outcome and risk factors. *Br J Ophthalmol* 2002; **86**: 536–542.
7 Seal DV, Beattie TK, Tomlinson A, Fan D, Wong E. *Acanthamoeba* keratitis in England and Wales: incidence,

outcome and risk factors. *Br J Ophthalmol (Mailbox)* 2003; **87**: 516–517.
8 Gail MH, Benichou J. *Encyclopedia of Epidemiologic Methods*. John Wiley & Sons: New York, 2000.
9 Houang E, Lam D, Fan D, Seal D. Microbial keratitis in Hong Kong — relationship to climate, environment and contact lens disinfection. *Trans R Soc Trop Med Hyg* 2001; **95**: 361–367.
10 Seal DV, Kirkness CM, Bennett HGB, Peterson M, Keratitis Study Group. *Acanthamoeba* keratitis in Scotland: risk factors for contact lens wearers. *Contact Lens Ant Eye* 1999; **22**: 58–68.
11 Yu HS, Choi KH, Kim HK, Kong HH, Chung DI. Genetic analysis of *Acanthamoeba* isolates from contact lens storage cases of students in Seoul, Korea. *Korean J Parasitol* 2001; **39**: 161–170.
12 Kong HH, Shin J-Y, Yu H-S, Kim J, Hahn T-W, Hahn Y-H *et al*. Mitochondrial DNA restriction fragment length polymorphism (RFLP) and 18S small-subunit ribosomal DNA PCR-RFLP analyses of *Acanthamoeba* isolated from contact lens storage cases of residents in Southwestern Korea. *J Clin Microbiol* 2002; **40**: 1199–1206.
13 Devonshire P, Munro FA, Abernethy C, Derbyshire B. Microbial contamination of contact lens cases in the West of Scotland. *Br J Ophthalmol* 1993; **77**: 41–45.
14 John DT. Opportunistically pathogenic free-living ameba. In: Baker JR, Kreier JP (eds). *Parasitic Protozoa*, 2nd edn. Academic Press: San Diego, CA, 1993, pp 143–246.
15 Martinez AJ, Visvesvara GS. Free-living amphizoic and opportunistic amoebas. *Brain Pathol* 1997; **7**: 583–598.
16 Pussard M, Pons R. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (*Protozoa, Amoebida*). *Protistologica* 1977; **8**: 557–598.
17 Visvesvara GS. Classification of *Acanthamoeba*. *Rev Infect Dis* 1991; **13**(S5): S369–S372.
18 Stothard DR, Schroeder-Diedrich JM, Awwad H, Gast RJ, Ledee DR, Rodriguez-Zaragoza S *et al*. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *J Eukaryotic Microbiol* 1998; **45**: 45–54.
19 Ledee D, Booton G, Sharma S, Byers TJ, Fuerst PA. The advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of *Acanthamoeba*. *Invest Ophthalmol Vis Sci* 2003; **44**: 1142–1149.
20 Yagita K, Endo T. Restriction enzyme analysis of mitochondrial DNA of *Acanthamoeba* strains in Japan. *J Protozool* 1990; **37**: 570–575.
21 Byers TJ, Hugo ER, Stewart VJ. Genes of *Acanthamoeba*: DNA, RNA and protein sequences (a review). *J Protozool* 1990; **37**: 17S–25S.
22 Kilvington S, Beeching JR, White DG. Differentiation of *Acanthamoeba* strains from infected corneas and the environment by using restriction endonuclease digestion of whole-cell DNA. *J Clin Microbiol* 1991; **29**: 310–314.
23 Gautom RK, Lory S, Seyedirashti S, Bergeron DL, Fritsche TR. Mitochondrial DNA fingerprinting of *Acanthamoeba* spp. isolated from clinical and environmental sources. *J Clin Microbiol* 1994; **32**: 1070–1073.
24 Chung DI, Kong HH, Yu HS, Oh Y-M, Yee ST, Lim YJ. Biochemical and molecular characterisation of a strain of KA/S2 of *Acanthamoeba castellanii* isolated from Korean soil. *Korean J Parasitol* 1996; **34**: 79–85.
25 Kong HH, Park JH, Chung DI. Interstrain polymorphisms of isoenzyme profiles and mitochondrial DNA fingerprints among seven strains assigned to *Acanthamoeba* polyphaga. *Korean J Parasitol* 1995; **33**: 331–340.
26 Chung DI, Yu H-S, Hwang M-Y, Kim T-H, Kim T-O, Yun H-C *et al*. Subgenus classification of *Acanthamoeba* by riboprinting. *Korean J Parasitol* 1998; **36**: 69–80.
27 Vodkin MH, Howe DK, Visvesvara GS, McLaughlin GL. Identification of *Acanthamoeba* at the generic and specific levels using the polymerase chain reaction. *J Protozool* 1992; **39**: 378–385.
28 Howe DK, Vodkin MH, Novak RJ, Visvesvara G, McLaughlin GL. Identification of two genetic markers that distinguish pathogenic and nonpathogenic strains of *Acanthamoeba*. *Parasitol Res* 1997; **83**: 345–348.
29 Lehmann MO, Green SM, Morlet N, Keys MF, Matheson MM, Dart JKG *et al*. Polymerase chain reaction analysis of corneal epithelial and tear samples in the diagnosis of *Acanthamoeba* keratitis. *Invest Ophthalmol Vis Sci* 1998; **39**: 1261–1265.
30 Schroeder JM, Booton GC, Hay J, Niszl I, Seal DV, Markus MB *et al*. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoebae* from humans with keratitis and sewage sludge. *J Clin Microbiol* 2001; **39**: 1903–1911.
31 Dykova I, Lom J, Schroeder-Diedrich JM, Booton GC, Byers TJ. *Acanthamoeba* strains isolated from organs of freshwater fishes. *J Parasitol* 1999; **85**: 1106–1111.
32 Booton GC, Kelly DJ, Chu Y-W, Seal D, Houang E, Lam D *et al*. 18S ribosomal DNA typing and tracking of *Acanthamoeba* sp. from corneal scrape specimens, contact lenses, lens cases and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *J Clin Microbiol* 2002; **40**: 1621–1625.
33 Stothard DR, Hay J, JSchroeder-Diedrich JM, Seal DV, Byers TJ. Fluorescent oligonucleotide probes for clinical and environmental detection of *Acanthamoeba* and the T4 18S rRNA gene sequence type. *J Clin Microbiol* 1999; **37**: 2687–2693.
34 Gast RJ. Development of an acanthamoeba-specific reverse dot-blot and the discovery of a new ribotype. *J Eukaryotic Microbiol* 2001; **48**: 609–615.
35 Schuster FL. Cultivation of pathogenic and opportunistic free-living amoebas. *Clin Microbiol Rev* 2002; **15**: 342–354.
36 Ficker L, Seal D, Warhurst D, Wright P. *Acanthamoeba* keratitis — resistance to medical therapy. *Eye* 1990; **4**: 835–838.
37 Hay J, Kirkness CM, Seal DV, Wright P. Drug-resistance and *Acanthamoeba* keratitis: the quest for alternative antiprotozoal chemotherapy. *Eye* 1994; **8**: 555–563.
38 Stapleton F, Seal DV, Dart JKG. Possible environmental sources of *Acanthamoeba* sp. that cause keratitis in contact lens wearers. *Rev Inf Dis* 1991; **13**(Suppl 5): S392.
39 Seal DV, Dart J, Stapleton F. Possible environmental sources of *Acanthamoeba* sp. in contact lens wearers. *Br J Ophthalmol* 1992; **76**: 424–427.
40 Ledee DR, Hay J, Byers TJ, Seal DV, Kirkness CM. *Acanthamoeba griffini*. Molecular characterization of a new corneal pathogen. *Invest Ophthalmol Vis Sci* 1996; **37**: 544–550.
41 Booton GC, Rogerson A, Davidian T, Seal D, Kelly D, Beattie T *et al*. Molecular and physiological evaluation of subtropical evironmental isolates of *Acanthamoeba* spp., causal agent of *Acanthamoeba* keratitis. 2003 (ARVO Presentation).

42 Beattie TK, Tomlinson A, McFadyen AK, Seal DV, Grimason A. Enhanced attachment of *Acanthamoeba* to extended-wear silicone hydrogel contact lenses: a new risk factor for infection. *Ophthalmology* 2003; **110**: 765–771.
43 de Jonckheere JF. Ecology of *Acanthamoeba*. *Rev Inf Dis* 1991; **13**(Suppl 5): S385–S387.
44 Sawyer TK, Lewis EJ, Galasso M, Lear DW, O'Malley ML, Adams WN *et al*. Pathogenic amoebae in ocean sediments near waste water sludge disposal sites. *J Water Pollution Control Federation* 1982; **54**: 1318–1323.
45 De Jonckheere J. Taxonomy. In: Ronadelli EG (ed.). *Amphizoic Amoebae. Human Pathology.* Piccin Nuova Libraria: Padova, Italy, 1987, pp 25–48.
46 Im KI. Pathogenic free-living amoebae. *Korean J Parasitol* 1990; **28**(Suppl): 29–39.
47 Moura H, Wallace S, Visvesvara GS. *Acanthamoeba healyi* n. sp. and the isoenzyme and immunoblot profiles of *Acanthamoeba* spp., Groups 1 and 3. *J Protozool* 1992; **39**: 573–583.
48 Walochnik J, Haller-Schober EM, Kolli H, Picker O, Obwaller A, Aspock H. Discrimination between clinically relevant and non-relevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria. *J Clin Microbiol* 2000; **38**: 3932–3936.
49 Walochnik J, Obwaller A, Aspock H. Correlation between morphological, molecular biological and physiological characteristics in clinical and non-clinical isolates of *Acanthamoeba* sp. *Appl Environ Microbiol* 2000; **66**: 4408–4413.
50 Walochnik J, Obwaller A, Aspock H. Immunological inter-strain cross-reactivity correlated to 18S rDNA sequence types in *Acanthamoeba* sp. *Int J Parasitol* 2001; **31**: 163–167.
51 Khan NA, Jarroll EL, Paget TA. Molecular and physiological differentiation between pathogenic and non-pathogenic *Acanthamoeba*. *Curr Microbiol* 2002; **45**: 197–202.
52 Wright P, Warhurst D, Jones BR. *Acanthamoeba* keratitis successfully treated medically. *Br J Ophthalmol* 1985; **69**: 778–782.
53 Seal DV, Hay J, Kirkness CM, Morrell A, Booth A, Tullo A *et al*. Successful medical therapy of *Acanthamoeba* keratitis with topical chlorhexidine and propamidine. *Eye* 1996; **10**: 413–421.
54 Larkin DFP, Kilvington S, Dart JKG. Treatment of *Acanthamoeba* keratitis with polyhexamethylene biguanide. *Ophthalmology* 1992; **99**: 185–191.
55 Duguid IGM, Dart JKG, Morlet N, Allan BDS, Matheson M, Ficker L *et al*. Outcome of *Acanthamoeba* keratitis treated with polyhexamethylene biguanide and propamidine. *Ophthalmology* 1997; **104**: 1587–1592.
56 Byers TJ, Akins RA, Maynard BJ, Lefken RA, Martin SM. Rapid growth of *Acanthamoeba* in defined media: induction of encystment by glucose-acetate starvation. *J Protozool* 1980; **27**: 216–219.
57 Elder MJ, Kilvington S, Dart JKG. A clinicopathologic study of *in vitro* sensitivity testing and *Acanthamoeba* keratitis. *Invest Ophthalmol Vis Sci* 1994; **35**: 1059–1064.
58 Burger RM, Franco RJ, Drlica K. Killing Acanthamoebae with polyaminopropyl biguanide: quantitation and kinetics. *Antimicrob Ag Chemother* 1994; **38**: 886–888.
59 Khunkitti W, Lloyd D, Furr JR, Russell AD. The lethal effects of biguanides on cysts and trophozoites of *Acanthamoeba castellanii*. *J Appl Bacteriol* 1996; **81**: 73–77.
60 Narasimhan S, Madhavan HN, Therese L. Development and application of an *in vitro* susceptibility test for *Acanthamoeba* species isolated from keratitis to polyhexamethylene biguanide and chlorhexidine. *Cornea* 2002; **21**: 203–205.
61 Perrine D, Chenu JP, Georges P, Lancelot JC, Saturnino C, Robba M. Amoebicidal efficiencies of various diamidines against two strains of *Acanthamoeba polyphaga*. *Antimicrobial Ag Chemother* 1995; **39**: 339–342.
62 Brasseur G, Favennec L, perrine D, Chenu JP, Brasseur P. Successful treatment of *Acanthamoeba* keratitis by hexamidine. *Cornea* 1994; **13**: 456–462.
63 Jennings FW. Future prospects for the chemotherapy of human trypanosomiasis. *Trans R Soc Trop Med Hyg* 1990; **84**: 618–621.
64 Jennings FW. Topical chemotherapy for experimental African trypanosomiasis with cerebral involvement: the use of melarsoprol combined with 5-nitroimidazole, megazol. *Trop Med Int Health* 1996; **1**: 363–366.
65 Jennings FW, Atouguia JM, Murray M. Topical chemotherapy for experimental murine African CNS-trypanosomiasis: the successful use of the arsenical melarosprol combined with the 5-nitroimidazoles, fexinidazole or MK-436. *Trop Med Int Health* 1996; **1**: 590–598.
66 de Koning HP. Transporters in African trypanosomes: role in drug action and resistance. *Int J Parasitol* 2001; **31**: 512–522.
67 Ledee D, Seal DV, Byers T. Confirmatory evidence from 18S rRNA gene analysis for *in vivo* development of propamidine resistance in a temporal series of *Acanthamoeba* ocular isolates from a patient. *Antimicrob Ag Chemother* 1998; **42**: 2144–2145.
68 Reinert KE. DNA multimode interaction with Berenil and pentamidine; double helix stiffening, unbending and bending. *J Biomol Struct Dyn* 1999; **17**: 311–331.
69 Gupta S, Srivastava DK, Shukla OP. Inhibition of polyamine metabolism induces amoebic encystation. *Curr Sci* 1986; **55**: 732–734.
70 Byers TJ, Kim BG, King LE, Hugo ER. Molecular aspects of the cell cycle and encystment of *Acanthamoeba*. *Rev Inf Dis* 1991; **13**(Suppl 5): S378–S384.
71 Hugo ER, Byers TJ. *S*-adenosyl-L-methionine decarboxylase of *Acanthamoeba castellanii* (Neff): purification and properties. *Biochem J* 1993; **295**: 203–209.
72 Ekelund S, Nygren P, Larsson R. Guanidino-containing drugs in cancer chemotherapy: biochemical and clinical pharmacology. *Biochem Pharmacol* 2001; **61**: 1183–1193.
73 Walochnik J, Duchene M, seifert K, Obwaller A, Hottkowitz T, Wiederman G *et al*. Cytotoxic activities of alkylphosphocholines against clinical isolates of Acanthamoeba spp. *Antimicrob Ag Chemother* 2002; **46**: 695–701.
74 Jha TK, Sundar S, Thakar CP, Bachmann P, Karbwarig J, Fisher C *et al*. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. *New Engl J Med* 1999; **341**: 1795–1800.
75 Guzek JP, Roosenberg JM, Gano DL, Wessels IF. The effect of vehicle on corneal penetration of triturated ketoconazole and itraconazole. *Ophthalmic Surg Lasers* 1998; **29**: 926–929.
76 Savani DV, Perfect JR, Cobo LM, Durack DT. Penetration of new azole compounds into the eye and efficacy in experimental *Candida* endophthalmitis. *Antmicrob Ag Chemother* 1987; **31**: 6–10.
77 Niederkorn JY, Alizadeh H, Leher H, McCulley JP. The pathogenesis of *Acanthamoeba* keratitis. *Microb Infect* 1999; **1**: 437–443.