1   *In vitro* evaluation of the inhibitory effect of topical ophthalmic agents
2   on *Acanthamoeba* viability
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17 **Running title**: Topical medications on the viability of Acanthamoeba spp.

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28 **ABSTRACT**

29 **Purpose:** To compare the antimicrobial effect of topical anaesthetics, antivirals, antibiotics,

30 and biocides on the viability of *Acanthamoeba* cysts and trophozoites *in vitro*.

31 **Methods:** Amoebicidal and cysticidal assays were performed against both trophozoites and

32 cysts of *A. castellanii* (ATCC 50370) and *A. polyphaga* (ATCC 30461). Test agents included

33 topical ophthalmic preparations of commonly anaesthetics, antivirals, antibiotics, and

34 biocides. Organisms were exposed to serial two-fold dilutions of the test compounds in the

35 wells of a microtitre plate to examine the effect on *Acanthamoeba* spp. In addition, the

36 toxicity of each of the test compounds was determined against a mammalian cell line.

37 **Results:** Proxymetacaine, oxybuprocaine and especially tetracaine were all toxic to the

38 trophozoites and cysts of *Acanthamoeba* but lidocaine was well tolerated. The presence of

39 benzalkonium chloride (BAC) preservative in levofloxacin caused a high level of toxicity to

40 trophozoites and cysts. With the diamidines the presence of BAC in the propamidine drops was

41 responsible for the activity against *Acanthamoeba*. Hexamidine drops without BAC showed

42 good activity against trophozoites and the biguanides PHMB, chlorhexidine, alexidine and

43 octenidine all showed excellent activity against trophozoites and cysts of both species.

44 **Conclusions:** The anti-amoebic effect of BAC, povidone iodine and tetracaine are superior to

45 the current diamidines and slightly inferior to the biguanides used in the treatment for

46 *Acanthamoeba* keratitis.

47 **Translational Relevance:** Ophthalmologists should be aware that certain topical anaesthetics

48 and ophthalmic preparations containing BAC, prior to specimen sampling may affect the

49 viability of *Acanthamoeba in vivo*, resulting in false negative results in diagnostic tests.

50

INTRODUCTION

Acanthamoeba is a genus of small free-living amoebae common to most soil and freshwater habitats.[1] The organism has a life cycle of a feeding and replicating trophozoite which, in response to adverse conditions, can form a dormant cyst stage.[1] *Acanthamoeba* spp. are opportunistic pathogens of humans causing a fatal granulomatous encephalitis (GAE) in the immunocompromised host and, more frequently, a potentially blinding keratitis in both non-contact lens (CL) or CL wearers.

Currently there are approximately 4.1 million contact lens wearers in the UK,[2] and established independent risk factors for developing acanthamoeba keratitis (AK) in CL wearers include: exposure to tap water in home, [3, 4] swimming or bathing when wearing CL, [4, 5] poor lens hygiene [4-6], and the use of rigid CL in orthokeratology.[6] Furthermore, previous outbreaks of AK in both the UK and USA and have been attributed to efficacy issue with certain contact lens disinfections system.[7, 8]

Despite the sight threatening risk with AK, in most series, it accounts for less than 5% of all CL related microbial keratitis. The reported incidence rates in CL users are 1 to 2 per million in the USA to 17 to 20 per million in the UK.[4] A recent study from a tertiary hospital in the UK reported a incidence rate of just 2.3% for *Acanthamoeba* over a 12-year period from over 1500 keratitis cases.[9] Due to the low number of patients with AK, many are diagnosed late due to initially being mis-diagnosed and treated for bacterial or other forms of keratitis such as fungal and herpes simplex keratitis.[4,10] Late diagnosis of AK has a massive impact on prognosis and patients are more likely to develop poorer visual outcome, longer duration of treatment, corneal perforation, and the requirement of penetrating keratoplasty.[10] Current medical therapy for AK is unlicensed and involves the topical administration of a biguanide either 0.02% polyhexamethylene biguanide (PHMB) or 0.02% chlorhexidine, either as

77 monotherapy or in combination with 0.1% propamidine or 0.1% hexamidine.[11] PHMB and

78 chlorhexidine have been reported to be the most effective and are effective against both

79 trophozoites and cysts of Acanthamoeba. [12-14]

80

81 In the UK, diagnosis of AK is not standardised and depends largely upon individual clinics

82 and hospitals. [15] A variety of methods can be employed with culture of a corneal scrape on

83 2.5% non-nutrient agar which has been overlaid with a lawn of *Escherichia coli*, the most

84 common method utilised and considered the gold standard. Despite the wide use of this

85 culture-based method, poor sensitivity means that in many cases the culture comes back

86 negative in patients with the infection. A recent study looking at diagnostic sensitivity

87 reported a value of 33.3% for culture compared to 74.1% and 100% for polymerase chain

88 reaction (PCR) and *in vivo* confocal microscopy, respectively.[16]

89

90 One possibility for the low sensitivity of culture-based diagnostics for *Acanthamoeba* could

91 be related to prior topical therapy such as anaesthetics and antibiotics applied to the cornea

92 prior to the corneal scrape being performed on the patient. Goldschmidt *et al* have found that

93 fluorescein and topical anaesthetics could interfere with real time PCR to detect herpes virus

94 and *Acanthamoeba* resulting in false negative results.[17] Other studies have also shown the use

95 of Rose Bengal and Lissamine green reduced PCR detection rate for herpes virus and

96 toxoplasma.[18] Furthermore, empirical antibiotic treatment with fluoroquinolone drugs or

97 other biocides prior to diagnosis could have an effect on the viability of *Acanthamoeba*.

98 Aside from the antimicrobial drugs, many ophthalmic preparations utilise benzalkonium

99 chloride (BAC) as a preservative and it has been shown BAC is highly toxic to

100 *Acanthamoeba*.[19]

101

102 Due to the potential effect of topical anaesthetics and antimicrobials on the viability of

103 *Acanthamoeba* cysts and trophozoites, we studied the activity of a range of commonly used

104 topical anaesthetics, antibiotics, antivirals and biocides against the trophozoite and cyst stage

105 of *A. polyphaga* (ATCC 30461) and *A. castellanii* (ATCC 50370), and a mammalian cell line.

106

107 **MATERIALS AND METHODS**

108 **Reagents and test compounds.**

109 We tested a range of ophthalmic preparations including biocides, diamidines, anaesthetics,

110 antivirals and antibiotics. All agents were obtained from Sigma Chemical Company Ltd

111 (Poole, UK) unless otherwise stated. The biocides, diamidines and miscellaneous compounds

112 included Brolene® (Propamidine isethionate 0.1% w/v, Sanofi, UK), Desomedine®

113 (Hexamidine di-isetionate 0.1%, Bausch & Lomb, France), PHMB (Lonza, UK), octenidine

114 (Schulke & Mayr, Germany), chlorhexidine digluconate 0.1% w/v, alexidine 0.1% w/v,

115 propamidine 0.1% w/v, hexamidine 0.1% w/v pentamidine 0.1% w/v, BAC 0.1% w/v,

116 phenylmercuric nitrate 0.1% w/v, fluorescein sodium 2% w/v (Minims, Bausch & Lomb,

117 UK) and povidone iodine 5% w/v (Minims, Bausch & Lomb, UK). Antibiotics used were

118 preserved levofloxacin 5 mg/ml (Oftaquix®, Santen, UK), preservative free levofloxacin

119 5mg/ml (Oftaquix Unit Dose®, Santen, UK), moxifloxacin 0.5% w/v (Moxeza®, Novartis,

120 UK), preserved chloramphenicol 0.5% w/v (Martindale Pharma, UK), and preservative free

121 chloramphenicol 0.5% w/v (Minims, Bausch & Lomb, UK). The topical anaesthetics tested

122 were all in MINIMS® formulation (Bausch & Lomb, UK) and they included proxymetacaine

123 (proparacaine) 0.5% w/v, tetracaine 1% w/v, oxybuprocaine 0.4% w/v, and lidocaine 4% w/v

124 with fluorescein sodium 0.25% w/v. Antivirals used were trifluorothymidine 1% w/v

125 (Stockport Pharmaceuticals, UK) and aciclovir 0.1% w/v. All the compounds were stored

126 according to the manufacturers' recommendations.

127

128

129

130 **Test organism strains and culture**.

131 *A. castellanii* (ATCC 50370) and *A. polyphaga* (ATCC 30461) were obtained from the

132 American Type Culture Collection (LGC standards, Teddington, UK). Trophozoites were

133 maintained in a semi-defined axenic broth medium as previously described.[20] Cysts were

134 produced using Neff's encystment medium (NEM) method as previously described [20].

135 Trophozoites were seeded into large tissue culture flasks (Nunc, UK) at a density of 1×10^5

136 cells / ml in 50 ml of growth medium and incubated for 48 hours at 30°C. The trophozoites

137 were harvested by centrifugation at 500 x *g* for 5 minutes and washed three times with ¼

138 strength Ringer's solution. The final pellet was then inoculated into 50 mL of NEM at a

139 density of 1×10^6 cells / ml into tissue culture flasks. The cultures were then incubated at

140 30°C for 7 days on a shaking incubator. The cysts were harvested for testing after 7 days of

141 incubation in NEM and washed three times with ¼ strength Ringer's solution. The pellet was

142 adjusted to 5×10^6 cysts / ml using a modified Fuchs Rosenthal haemocytometer (Hawksley,

143 UK) and the cysts stored at 4 to 8°C for testing within 14 days.

144

145 **Amoebicidal assays.**

146 In the trophozoite assay, serial two-fold dilutions of the test compounds were made in the

147 wells of a tissue culture grade microtitre plate (Helena Biosciences, Gateshead, UK).

148 Ophthalmic preparation used straight from the bottle and serially diluted from the

149 concentration stated on the product information label. Pure drugs were prepared as 1 mg/ml

150 (0.1%) stock solution in an appropriate solvent. Control wells received ¼ strength Ringer's

151 solution in place of test solution. Log phase cultures of axenic trophozoites were adjusted to a

152 concentration of 2×10^4 /ml in growth medium and 100 µl of the calibrated suspension added

153 to the wells for incubation at 30°C in triplicate. After 48 h, the wells were inspected using an

154 inverted microscope. This was achieved by comparing the appearance of the trophozoites in

155 the test wells to those in controls. Typically this involves visually comparing the degree of

156 amoeba growth relative to the control as well as looking for cell lysis and rounding of the

157 amoebae. The minimum trophozoite inhibitory concentration (MTIC) was defined as 50 %

158 inhibition of *Acanthamoeba* trophozoite replication compared to the controls. The minimum

159 trophozoite amoebicidal concentration (MTAC) was defined as the lowest concentration of

160 test compound that resulted in the complete lysis or degeneration of the trophozoites.

161

162 **Cysticidal assays.**

163 The cysticidal assay relies on the observation that *Acanthamoeba* cysts adhere to the well

164 bottoms of the microtitre plates and remain attached following exposure to the test compound

165 and removal by washing. Addition of a live *E. coli* to the wells, followed by incubation,

166 results in encystment of viable cysts and replication of the emergent trophozoites. Serial two-

167 fold dilutions of the test compounds were prepared with distilled water in the wells of the

168 microtitre plate. Cysts were adjusted to a final concentration of 2×10^4 cells / ml in ¼

169 strength Ringer's solution and 100 µl added to each well. The plates were then incubated at

170 30°C for 48 hours. After incubation the wells were aspirated to remove the drug using a

171 Vacusip (Integra, UK) and refilled with ¼ strength Ringer's solution. This process was

172 repeated three times to ensure removal of the drugs from the wells. After the final aspiration

173 the wells were filled with 100 µl of ¼ strength Ringer's containing live *E. coli* (ATCC 8739)

174 at an optical density of 0.2 at 540nm and incubated at 30°C. The minimum cysticidal

175   concentration (MCC) was defined as the lowest concentration of test compound that resulted

176   in no excystation and trophozoite replication after 7 days incubation.

177

178

179

180   **Hep-2 cell cytotoxicity.**

181   The cytotoxicity of the test compounds was determined against the Hep-2 (HeLa derivative)

182   human cervix carcinoma cell line (ECACC #86030501) obtained from the European

183   Collection of Cell Cultures (Centre for Applied Microbiology and Research, Salisbury, U.K).

184   The cells were grown and maintained at 37°C in Minimum Essential Medium with 10% heat-

185   inactivated foetal bovine serum (Life Technologies Ltd, Paisley, Scotland). Flasks containing

186   confluent monolayers of cells were used to seed a 96 well microtitre plate at a concentration

187   of 1×10^4 cells /well in 100 µl of growth medium with incubation at 37°C. Once

188   approximately 75% confluent growth occurred in the wells, the medium was changed, and

189   the cells used for cytotoxicity testing. Serial two-fold dilutions of the test agent in appropriate

190   solvent were added to the wells and the plate incubated at 37°C for 24 hours. The degree of

191   cytotoxicity was determined using the CellTiter 96® AQ$_{uous}$ One Solution Cell Proliferation

192   Assay (Promega, Southampton, U.K.). This is a colourmetric assay in which metabolically

193   active cells bioreduce a tetrazolium compound to generate a soluble coloured formazan

194   product whose abundance can be measured spectrophotometrically at 595nm [21].

195

196   **Transmission Electron Microscopy (TEM) of *Acanthamoeba* cysts**

197   For the TEM studies Neff's cysts of *A. castellanii* were used. The cysts were exposed to the

198   test formulations using either topical ophthalmic preparations (tetracaine 1% and preserved

199   chloramphenicol 0.5%) or a solutions made up to the same concentration used in ophthalmic

200 preparations (PHMB 0.02%, unpreserved chloramphenicol 0.5%, benzalkonium chloride 0.05

201 mg/ml and povidone-iodine 5%). Controls cysts were exposed to 1/4 strength Ringer's

202 solution. The cysts were exposed to the test formulations at 32 °C for 1 hour. The agents were

203 removed by washing the cysts with ¼ strength Ringer's solution and centrifuged at 1000 x g

204 for 5 minutes. The resulting pellets were fixed with 2.5% (v/v) glutaraldehyde buffered with

205 0.1M HEPES at pH 7.2 overnight at 4°C before being processed for TEM microscopy.

206

207 **RESULTS**

208 The activity of the test compounds against the trophozoites and cysts of *A. castellanii* (ATCC

209 50370) and *A. polyphaga* (ATCC 30461) and their toxicity for Hep-2 cells is shown in Tables

210 1-4.

211

212 The results for the topical anaesthetics and fluorescein sodium are shown in Table 1. The

213 inhibitory range against *Acanthamoeba* trophozoites for the anaesthetics proxymetacaine,

214 tetracaine and oxybuprocaine were 9.75-39 µg/ml whereas lidocaine produced no inhibition

215 of growth until the 312-625 µg/ml range for both species. In the trophozoite amoebicidal

216 studies proxymetacaine, tetracaine and oxybuprocaine were amoebicidal in the 19.5-250

217 µg/ml range whereas with lidocaine, the amoebicidal activity against *A. polyphaga* and *A.*

218 *castellanii* was 312 and 1250 µg/ml, respectively. For the cyst assays, proxymetacaine,

219 tetracaine and oxybuprocaine were cysticidal in the 39-250 µg/ml range. With lidocaine the

220 cysticidal activity against *A. polyphaga* and *A. castellanii* was 1.25 and 10 mg/ml,

221 respectively. In the toxicity assay against the mammalian cell line, proxymetacaine, tetracaine

222 and oxybuprocaine were cytotoxic in the 39-156 µg/ml range whereas lidocaine produced no

223 cytotoxicity until 5 mg/ml. The lidocaine MINIMS contain fluorescein sodium and so as a

224   control this was tested separately and found to be non-toxic at the 2% concentration (Table

225   1).

226

227

228

229   The results for the topical antibiotics, antivirals and preservatives are shown in Table 2. For

230   the fluoroquinolones, levofloxacin (Oftaquix®) formulation demonstrated trophozoite

231   inhibitory and amoebicidal activity in the 78-312 µg/ml range whereas for the unpreserved

232   levofloxacin (pure drug without BAC), the values were in the 312 to 1250 µg/ml range for

233   both species, a factor of 4 difference in magnitude from the commercial ophthalmic

234   preparation. Moxifloxacin (Moxeza®) demonstrated trophozoite inhibitory and amoebicidal

235   activity in the 625-2500 µg/ml range for both species. Against cysts, levofloxacin

236   (Oftaquix®) was cysticdal at 625 µg/ml compared to levofloxacin (pure drug) and

237   moxifloxacin which showed cysticidal activity in the 2.5-5 mg/ml range. For the toxicity

238   assay, levofloxacin (Oftaquix®) and moxifloxacin gave a toxicity of 39 and 156 µg/ml,

239   respectively. Two preparation of the chloramphenicol were tested. The preserved and

240   preservative free versions showed inhibitory activity against trophozoites at 39 to 312 µg/ml

241   whereas for the cysts this ranged from 312 µg/ml to 2.5 mg/ml for the two species of

242   *Acanthamoeba*, respectively. Phenylmercuric nitrate which is the preservative used in

243   Chloramphenicol was active against trophozoites in the 1-3.9 µg/ml range and against cysts

244   in the 15.6-31.3 µg/ml range. BAC which is commonly added to ophthalmic preparations as a

245   preservative was active against trophozoites in the 1-7.8 µg/ml range and against cysts in the

246   7.8-15.6. µg/ml range.

247

248 The antiviral Trifluorothymidine (TFT) was active against trophozoites in the 312-1250

249 µg/ml range and against cysts in the 2.5-5 mg/ml range. Aciclovir ophthalmic ointment

250 (Zovirax®) could not be used due to the soft paraffin base and so a solution was prepared

251 from pure drug. Aciclovir was active against trophozoites in the 63-250 µg/ml range but

252 showed no activity against cysts in the range tested.

253

254 The effect of the diamidines against *Acanthamoeba* trophozoites and cysts and the toxicity to

255 the mammalian cell line is shown in Table 3. With hexamidine, the pure drug and the

256 Desomedine® formulation performed identically in all tests against both species and the

257 mammalian cell line. With propamidine, the pure drug showed a trophozoite inhibitory effect

258 in the 63-250 µg/ml range compared to 7.8-15.6 µg/ml range for the Brolene® formulation.

259 For the amoebicidal activities, propamidine (pure drug) was in the 250-500 µg/ml range

260 compared to 15.6-31.3 µg/ml range for the Brolene® formulation. Both the pure drug and

261 Brolene® formulation showed limited to no activity against the cysts of both species. In the

262 toxicity studies, the propamidine (pure drug) showed limited toxicity at 250 µg/ml compared

263 to 31.3 µg/ml for the Brolene® formulation. Pentamidine showed almost identical activity to

264 that of propamidine. Comparatively, BAC was more effective than any of the diamidines

265 tested showing activity against the trophozoites, cysts, and greater cytotoxicity to mammalian

266 cells (Table 2).

267

268 The effect of the biguanide compounds and povidone iodine against *Acanthamoeba*

269 trophozoites and cysts, and the toxicity to the mammalian cell line is shown in Table 4. In the

270 trophozoite assay all of the biguanides demonstrated inhibitory activity in the 0.5-1.95 µg/ml

271 range and amoebicidal activity in the 1-15.6 µg/ml range. For cysticidal activity, the

272 biguanides ranged from 3.9-31.3µg/ml; both octenidine and Alexidine were comparable to

273 PHMB in terms of antimicrobial efficacy. For the toxicity assay, the biguanides ranged

274   between 1-31.3 µg/ml with PHMB demonstrating the highest value (least cytotoxic).

275   Povidone Iodine was active against trophozoites in the 7.8-31.3 µg/ml range and against cysts

276   in the 7.8-15.6 µg/ml range.

277

278

279

280   The changes in the morphology of the cysts when exposed to various compounds taken with

281   TEM are shown in Figure 1. As a control, a healthy Neff's cyst in ¼ strength Ringer's

282   solution can be seen in Figure 1A. The healthy cyst has a thick cyst wall surrounding the

283   encysted trophozoite. The trophozoite plasma membrane touches the endocyst wall taking up

284   the full space available inside the cyst. In the cytoplasm, the nucleus can clearly be seen as

285   can the rounded structures including mitochondria and lysosomes.

286

287   After exposure to tetracaine, the nucleus is no longer visible and the cytoplasm is full of

288   micelles caused by the breakup of the nuclear membrane (Figure 1B). With preserved

289   chloramphenicol, propamidine pure drug, and unpreserved chloramphenicol, no changes in

290   intracellular organisation of the cytoplasm and nucleus were observed, respectively (Figure

291   1C, D, and H).

292

293   With BAC (0.05 mg/ml), the plasma membrane of the encysted trophozoite has been

294   damaged and shrunk away from the walls of the endocyst and there is an lack of a defined

295   nucleolus and an increase in the number of cytoplasmic micelles consistent with membrane

296   damage (Figure 1E).

297

298 With povidone iodine (5% w/v) and PHMB, the plasma membrane of the encysted

299 trophozoite has been severely damaged and has shrunk significantly away from the walls of

300 the endocyst. No defined nuclear structures were seen and there are large numbers of micellar

301 aggregations inside the cyst suggesting complete plasma membrane destruction in the

302 encysted trophozoite (Figure 1F and 1G).

303

304

305

306 **DISCUSSION**

307 AK is a sight-threatening corneal infection and early diagnosis is paramount in achieving a

308 better prognosis and visual outcome. Risk factor determination and clinical examination can

309 often differentiate AK from other forms of keratitis, with ring infiltrate or disease confined to

310 the epithelium to be more common compared to bacterial and fungal keratitis.[22] However, in

311 epithelial disease, especially associated with dendritic-type lesions, *Acanthamoeba* can be

312 misdiagnosed with other causes of keratitis such as herpes simplex (HSV) keratitis.[4,10]

313 Traditionally, cultures have been the mainstay in diagnosing *Acanthamoeba* but a low culture

314 positive rate and [23] prolonged incubation period, often lead to a delay in diagnosis and

315 treatment.[24] The use of IVCM and PCR in diagnosing AK have shown good promise with

316 sensitivity values ranging between 56-100% [25-28] and 77-88%, respectively.[16, 29, 30] The main

317 advantages of IVCM are it is non-invasive and it provides a rapid diagnosis but the main

318 limitations are the potential difficulty in differentiating pathogenic organisms from host cells,

319 and the diagnostic accuracy is dependent on observer experience.[26] PCR testing is quicker

320 and more sensitive than culture, returning a result often within days rather than weeks but

321 similar to cultures, false negative results do occur. Possible factors include the amount of

322 viable acanthamoeba obtained from the corneal scrape or biopsy and inhibition effect from

323 the use of topical agents before microbiological sampling such as prior empirical treatment

324 with antibiotics, the use of anaesthetics and vital stains such as fluorescein.[17, 31] There are

325 limited data on the inhibitory effect of topical anaesthetics with one study showing

326 proparacaine (proxymetacaine) did not adversely affect PCR [31] whereas in a second study,[17]

327 they found oxybuprocaine inhibited real-time PCR in detecting *Acanthamoeba*. We have

328 found the type of topical anaesthetic greatly affected the viability of *Acanthamoeba* in that

329 lidocaine had a much lower antimicrobial effect and at therapeutic concentration, it did not

330 exert significant antimicrobial activity against cysts and trophozoites for two species of

331 *Acanthamoeba*. Although the lidocaine used in this study was combined with fluorescein, we

332 did not find testing fluorescein on its own, had any major antimicrobial effect on the

333 trophozoites or cysts. This contrasts with the other topical anaesthetics, in particular

334 tetracaine which was observed to exert a much stronger antimicrobial effect against

335 trophozoites, cysts of *Acanthamoeba* and toxicity to a human cell line. The antimicrobial

336 effect of proxymetacaine was similar to oxybuprocaine for *A. castellanii* but for *A.*

337 *polyphaga*, proxymetacaine had a greater amoebicidal and cysticidal effect, in addition to

338 being more toxic to the human cell line. These results suggest the use of topical anaesthetics,

339 especially with tetracaine, can have potent anti-amoebic effect and it may be an important

340 contributory factor in the reported low sensitivity for culture from corneal scrape.

341

342 Depending on antibiotics protocol used, empirical treatment with a third or fourth generation

343 fluoroquinolones, due to their broad-spectrum antimicrobial activity, is often prescribed as an

344 initial therapy for the treatment of microbial keratitis.[32] We found the pure drug of

345 levofloxacin and a preservative free preparation of moxifloxacin (Moxeza®) did not exert any

346 major antimicrobial effect on the viability of *Acanthamoeba* whereas for preserved

347 levofloxacin (Oftaquix®), which is preserved with BAC, had a much greater antimicrobial

348 activity on both species of *Acanthamoeba*. This indicates it is the BAC in preserved

349 levofloxacin rather than the drug itself that is causing the antimicrobial effect observed.

350 Thompson and co-workers did not find any adverse effect on PCR amplification for

351 *Acanthamoeba* with gatifloxacin or moxifloxacin.[31] The gatifloxacin used in their study

352 (Zymar; Allergan, Irvine, CA) was preserved with BAC whereas the moxifloxacin was self-

353 preserved. Although they did not test BAC on its own, the minimal inhibitory effect found

354 with both antibiotics suggests the effect of BAC on PCR in detecting *Acanthamoeba* DNA

355 may be less compared to the amoebicidal and cysticidal assay methods used in this study.

356 Comparing the two preparations of chloramphenicol, the preserved drug had a much higher in

357 vitro activity against *Acanthamoeba* suggesting the anti-amoebic effect is related to the

358 preservative phenylmercuric nitrate. That said, the TEM images did not show much

359 difference in the morphology with the preserved and preservative free version of

360 chloramphenicol.

361 This difference in anti-amoebic activity was also seen when comparing preserved

362 propamidine (Brolene®), a common over the counter (OTC) ant-infective ophthalmic

363 preparation in the UK, with the pure drug propamidine. Previously, it has been shown that the

364 concentrations of BAC typically found in ophthalmic medicines are highly toxic to

365 *Acanthamoeba* trophozoites. In the study by Tu et al, exposing trophozoites to BAC

366 concentrations in the 10-30 µg/ml range, produced up to a 4.5 log reduction in viability over

367 6.5 hours.[19] However, this present study is the first to observe the effect of BAC containing

368 preparations against the highly resistant cystic stage of *Acanthamoeba*. We found the MTIC,

369 MTAC and MCC for BAC was significantly lower than the concentration of the BAC present

370 in both Brolene® (50 µg/ml) and Oftaquix® (50 µg/ml) ophthalmic preparations. Indeed, this

371 study has demonstrated that the presence of the BAC preservative in propamidine (Brolene®)

372 eye drops is likely to be solely responsible for the observed anti-amoebic activity. Two

373 previous studies have reported MTAC values for propamidine of 5-25 µg/ml and 0.49-0.97

374 μg/ml but in both studies they only used the benzalkonium preserved Brolene® without

375 comparing it to propamidine pure drug.[33, 34] This is the first study that has compared

376 propamidine (pure drug) against BAC preserved propamidine (Brolene®), and we found the

377 MTAC range for Brolene® (15.6-31.3 μg/ml) to be similar to the study found by Hay et al

378 and higher than the values found in the study by Elder et al. However, when tested in the

379 absence of BAC, the MTAC was much higher in the 250-500 μg/ml range (Table 2). This

380 confirms that the anti-amoebic activity of Brolene® is down to the presence of BAC in the

381 formulation. In fact, the amount of BAC in Brolene® (50 μg/ml) is much higher than the

382 MTAC found in this study, typically12.8 times greater than the concentration observed to

383 inhibit trophozoites. Morphologically, the TEM images shown clear evidence that BAC

384 causes damage to the cysts and this is in agreement with the findings from Sunada et al who

385 found destruction of the cytoplasmic elements and separation of the inner and outer walls

386 when cysts were exposed to BAC.[36] However, in their study, the TEM cyst images with BAC

387 and propamidine showed similar level of destruction of encysted trophozoite and this is due

388 to the fact that the authors did not use pure propamidine and instead they used GoldenEye®

389 eye drops which contain 0.1% w/v propamidine preserved with 0.05% benzalkonium

390 chloride.

391

392 Many multi-dose ophthalmic preparations utilise BAC as a preservative to prevent

393 contamination of the formulation, therefore, the empirical use of BAC preserved eye drops

394 prior to a diagnosis of AK is likely to have a negative impact on the viability of

395 *Acanthamoeba* present on the cornea, which may further contribute to the reported low

396 positive culture rate for AK from corneal scrapes. Aciclovir demonstrated greater inhibitory

397 and amoebicidal effect on trophozoites compared to trifluorothymidine but neither drug

398 appeared to be effective against the cysts. Aciclovir is commonly used to treat HSV keratitis

399 and as AK is often misdiagnosed as HSV, the potential inhibitory effect on

400 *Acanthamoeba*.trophozoites would need to be considered.

401

402 When the experiments were repeated with hexamidine (Desomedine®), another diamidine

403 commonly used in the treatment of AK, we found that this gave identical results to

404 hexamidine (pure drug). This result is not surprising as Desomedine®, unlike Brolene®, does

405 not contain any preservatives. Moreover, this study has examined the activity of three

406 different diamidines with increasing alkyl chain lengths between the aromatic benzene rings.

407 The observation that the 6 carbon hexyl chain length compound hexamidine has superior

408 activity to the propyl and pentyl diamidines, is consistent with a previous study in that

409 diamidine anti-amoebic activity increases with lipophilicity due to increased interaction with

410 the *Acanthamoeba* lipid bilayer.[35]

411

412 The aim of this study was to assess the potential inhibitory effect of prior administration of

413 commonly used topical agents on the viability of *Acanthamoeba* before microbiological

414 sampling but to obtain a sense of how potent these agents are, we also compared the *in vitro*

415 susceptibility to current and potential new treatment for AK. The results obtained in this

416 study for chlorhexidine and PHMB are consistent with previous published studies, with

417 PHMB demonstrating superior activity to chlorhexidine.[31, 36] The standard topical PHMB

418 used in treating AK is 0.02% (200 µg/ml) which is nearly 20 times the mean MCC for PHMB

419 found in this study, the finding agrees with the general favourable *in vitro* sensitivities and

420 clinical outcome with PHMB compared to other anti-amoebic drugs.[15] However, Sunada et al

421 did not find their *Acanthamoeba* isolates had high *in vitro* susceptibility to PHMB.[37]

422 Furthermore, the TEM appearance of the cysts in this study seem to be more affected with

423 damage to the cyst plasma membrane clearly seen compared to Sunada et al who found

424   mainly a loss of electron-dense material in the cytoplasm.[36] We found both alexidine and

425   octenidine demonstrated very good *in vitro* sensitivities and in fact octenidine was superior to

426   PHMB for MTAC and MCC for both species of *Acanthamoeba*, though toxicity against

427   mammalian cell line was comparatively higher. This is the first study that has reported the

428   activity of octenidine against *Acanthamoeba* cysts and trophozoites. The results of this study

429   on alexidine agree with a previous study which reported an MTAC of 10 μg/ml although they

430   observed a much higher MCC (100 μg/ml).[38] The exact reason for this difference is unclear

431   but the concentration of cysts used in the Alizadeh et al study was 100-fold greater at 1×10^{6}

432   compared to 1×10^{4} / ml used in this and other published studies. Neither alexidine and

433   octenidine are currently used to treat AK in a clinical setting but the favourable antimicrobial

434   activity against both *Acanthamoeba* cysts and trophozoites, especially with octenidine,

435   warrants further investigation as they may be useful in patients who do not respond to

436   standard antiamoebic treatment. Alexidine, at a concentration of 1.6 μg/ml, is incorporated in

437   one contact lens solution (AMO® RevitaLens OcuTec) and it has been demonstrated that

438   alexidine has excellent activity against cysts of *Acanthamoeba*.[39] Aside from Alexidine, the

439   majority of the multi-purpose contact lens solutions (MPS) utilises PHMB alone or in

440   conjunction with polyquaternium-1. The concentration of PHMB containing MPS are in the

441   order of 1 μg/ml, which is equivalent to the MTIC found in this study. Accordingly, the aim

442   of these MPS is to provide effective disinfectant properties through the prevention and

443   inhibition of pathogenic organisms on the contact lens or the contact lens case and therefore,

444   it is possible that the release of the biocide from the contact lens can exert and inhibitory

445   effect on the viability of *Acanthamoeba* on the cornea. The uptake and release of biocides

446   occurs in all types of contact lenses but the interaction is complex and varies with numerous

447   factors, therefore the potential inhibitory effect on *Acanthamoeba* with the release of biocide

448   from contact lenses on the cornea is currently unknown.

449

450   We found povidone iodine, as in other studies, demonstrated good *in vitro* activity on

451   *Acanthamoeba* isolates with clear signs of damage from the TEM images. In fact, the MCC

452   activity was identical to PHMB and superior to both propamidine and hexamidine. Although

453   povidone iodine is not routinely used prior to performing a cornea scrape, the potential

454   inhibitory effect on the viability of *Acanthamoeba* must be bore in mind.

455

456   The main limitation of this study is findings from *in vitro* sensitivities do not always correlate

457   with clinical outcome.[15] There are many factors to explain this. The cysticidal assay exposed

458   the cysts to the testing compound and then re-examined in 7 days for excystation and

459   trophozoite replication. In a clinical setting, corneal scrape for culture is performed straight

460   after the instillation of topical anaesthetics or vital stain, therefore, a shorter assay period

461   would be more representative. Although we tested two commonly used fluoroquinolones and

462   chloramphenicol, prior empirical antimicrobial treatment for microbial keratitis varies with

463   different institutions so the in vitro sensitivities might be different with different antibiotics.

464   In addition, the interaction of the epithelium with a drug and the penetration of it into the

465   stroma would be different with different type of drugs. These are shortcomings with *in vitro*

466   sensitivity and efficacy studies of drugs on pathogens. Notwithstanding this, the potential

467   adverse effect on the viability of *Acanthamoeba* with drugs used during clinical examination

468   to reduce pain or the prior treatment with antibiotics before obtaining tissue specimens for

469   culture or PCR has to be considered.

470

471   In conclusion, the present work indicates the use of proxymetacaine, oxybuprocaine and

472   tetracaine to reduce pain, ophthalmic preparations containing preservatives such as BAC and

473   the use of povidone iodine, prior to specimen sampling, may affect the viability of

Acanthamoeba in vivo, resulting in reduce culture yield and inhibition effect on PCR amplification.

Conflict of Interest: All authors have nothing to declare

REFERENCES

1. Marciano-Cabral F, Cabral G. Acanthamoeba spp. as agents of disease in humans. *Clin Microbiol Rev* 2003;16:273-307.

2. ACLM. The ACLM Contact Lens Year Book 2016. *The Association of Contact Lens Maufacturers* 2016.

3. Kilvington S, Gray T, Dart J, et al. Acanthamoeba keratitis: the role of domestic tap water contamination in the United Kingdom. *Invest Ophthalmol Vis Sci* 2004;45:165-169.

4. Radford CF, Minassian DC, Dart JK. Acanthamoeba keratitis in England and Wales: incidence, outcome, and risk factors. *Br J Ophthalmol* 2002;86:536-542.

5. Stehr-Green JK, Bailey TM, Brandt FH, Carr JH, Bond WW, Visvesvara GS. Acanthamoeba keratitis in soft contact lens wearers. A case-control study. *Jama* 1987;258:57-60.

6. Cope JR, Collier SA, Schein OD, et al. Acanthamoeba Keratitis among Rigid Gas Permeable Contact Lens Wearers in the United States, 2005 through 2011. *Ophthalmology* 2016;123:1435-1441.

7. Radford CF, Bacon AS, Dart JK, Minassian DC. Risk factors for acanthamoeba keratitis in contact lens users: a case-control study. *BMJ* 1995;310:1567-1570.

8. Verani JR, Lorick SA, Yoder JS, et al. National outbreak of Acanthamoeba keratitis associated with use of a contact lens solution, United States. *Emerg Infect Dis* 2009;15:1236-1242.

9. Tan SZ, Walkden A, Au L, et al. Twelve-year analysis of microbial keratitis trends at a UK tertiary hospital. *Eye (Lond)* 2017;31:1229-1236.

10. Carnt N, Robaei D, Minassian DC, Dart JKG. Acanthamoeba keratitis in 194 patients: risk factors for bad outcomes and severe inflammatory complications. *Br J Ophthalmol* 2018.

11. Dart JK, Saw VP, Kilvington S. Acanthamoeba keratitis: diagnosis and treatment update 2009. *Am J Ophthalmol* 2009;148:487-499 e482.

12. Lim N, Goh D, Bunce C, et al. Comparison of polyhexamethylene biguanide and chlorhexidine as monotherapy agents in the treatment of Acanthamoeba keratitis. *Am J Ophthalmol* 2008;145:130-135.

13. Lorenzo-Morales J, Martin-Navarro CM, Lopez-Arencibia A, Arnalich-Montiel F, Pinero JE, Valladares B. Acanthamoeba keratitis: an emerging disease gathering importance worldwide? *Trends in parasitology* 2013;29:181-187.

531   14.   Polat ZA, Walochnik J, Obwaller A, Vural A, Dursun A, Arici MK. Miltefosine and
532   polyhexamethylene biguanide: a new drug combination for the treatment of Acanthamoeba
533   keratitis. *Clin Exp Ophthalmol* 2014;42:151-158.

534   15.   Alexander CL, Coyne M, Jones B, Anijeet D. Acanthamoeba keratitis: improving the
535   Scottish diagnostic service for the rapid molecular detection of Acanthamoeba species. *J Med*
536   *Microbiol* 2015;64:682-687.

537   16.   Goh JWY, Harrison R, Hau S, Alexander CL, Tole DM, Avadhanam VS. Comparison
538   of In Vivo Confocal Microscopy, PCR and Culture of Corneal Scrapes in the Diagnosis of
539   Acanthamoeba Keratitis. *Cornea* 2018;37:480-485.

540   17.   Goldschmidt P, Rostane H, Saint-Jean C, et al. Effects of topical anaesthetics and
541   fluorescein on the real-time PCR used for the diagnosis of Herpesviruses and Acanthamoeba
542   keratitis. *Br J Ophthalmol* 2006;90:1354-1356.

543   18.   Seitzman GD, Cevallos V, Margolis TP. Rose bengal and lissamine green inhibit
544   detection of herpes simplex virus by PCR. *Am J Ophthalmol* 2006;141:756-758.

545   19.   Tu EY, Shoff ME, Gao W, Joslin CE. Effect of low concentrations of benzalkonium
546   chloride on acanthamoebal survival and its potential impact on empirical therapy of
547   infectious keratitis. *JAMA Ophthalmol* 2013;131:595-600.

548   20.   Kilvington S, Lam A. Development of standardized methods for assessing biocidal
549   efficacy of contact lens care solutions against Acanthamoeba trophozoites and cysts. *Invest*
550   *Ophthalmol Vis Sci* 2013;54:4527-4537.

551   21.   Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble
552   tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 1991;3:207-
553   212.

554   22.   Mascarenhas J, Lalitha P, Prajna NV, et al. Acanthamoeba, fungal, and bacterial
555   keratitis: a comparison of risk factors and clinical features. *Am J Ophthalmol* 2014;157:56-
556   62.

557   23.   Yeh DL, Stinnett SS, Afshari NA. Analysis of bacterial cultures in infectious keratitis,
558   1997 to 2004. *Am J Ophthalmol* 2006;142:1066-1068.

559   24.   Bharathi MJ, Ramakrishnan R, Meenakshi R, Mittal S, Shivakumar C, Srinivasan M.
560   Microbiological diagnosis of infective keratitis: comparative evaluation of direct microscopy
561   and culture results. *Br J Ophthalmol* 2006;90:1271-1276.

562   25.   Chidambaram JD, Prajna NV, Larke NL, et al. Prospective Study of the Diagnostic
563   Accuracy of the In Vivo Laser Scanning Confocal Microscope for Severe Microbial Keratitis.
564   *Ophthalmology* 2016;123:2285-2293.

565   26.   Hau SC, Dart JK, Vesaluoma M, et al. Diagnostic accuracy of microbial keratitis with
566   in vivo scanning laser confocal microscopy. *Br J Ophthalmol* 2010;94:982-987.

567   27.   Tu EY, Joslin CE, Sugar J, Booton GC, Shoff ME, Fuerst PA. The relative value of
568   confocal microscopy and superficial corneal scrapings in the diagnosis of Acanthamoeba
569   keratitis. *Cornea* 2008;27:764-772.

570   28.   Kanavi MR, Javadi M, Yazdani S, Mirdehghanm S. Sensitivity and specificity of
571   confocal scan in the diagnosis of infectious keratitis. *Cornea* 2007;26:782-786.

572   29.   Mathers WD, Nelson SE, Lane JL, Wilson ME, Allen RC, Folberg R. Confirmation
573   of confocal microscopy diagnosis of Acanthamoeba keratitis using polymerase chain reaction
574   analysis. *Archives of ophthalmology* 2000;118:178-183.

575   30.   Pasricha G, Sharma S, Garg P, Aggarwal RK. Use of 18S rRNA gene-based PCR
576   assay for diagnosis of acanthamoeba keratitis in non-contact lens wearers in India. *Journal of*
577   *clinical microbiology* 2003;41:3206-3211.

578   31.   Thompson PP, Kowalski RP, Shanks RM, Gordon YJ. Validation of real-time PCR
579   for laboratory diagnosis of Acanthamoeba keratitis. *Journal of clinical microbiology*
580   2008;46:3232-3236.

581   32.   Marangon FB, Miller D, Alfonso EC. Impact of prior therapy on the recovery and
582   frequency of corneal pathogens. *Cornea* 2004;23:158-164.

583   33.   Elder MJ, Kilvington S, Dart JK. A clinicopathologic study of in vitro sensitivity
584   testing and Acanthamoeba keratitis. *Invest Ophthalmol Vis Sci* 1994;35:1059-1064.

585   34.   Hay J, Kirkness CM, Seal DV, Wright P. Drug resistance and Acanthamoeba
586   keratitis: the quest for alternative antiprotozoal chemotherapy. *Eye* 1994;8 (Pt 5):555-563.

587   35.   Perrine D, Chenu JP, Georges P, Lancelot JC, Saturnino C, Robba M. Amoebicidal
588   efficiencies of various diamidines against two strains of Acanthamoeba polyphaga.
589   *Antimicrobial agents and chemotherapy* 1995;39:339-342.

590   36.   Kilvington S, Hughes R, Byas J, Dart J. Activities of therapeutic agents and
591   myristamidopropyl dimethylamine against Acanthamoeba isolates. *Antimicrobial agents and*
592   *chemotherapy* 2002;46:2007-2009.

593   37.   Sunada A, Kimura K, Nishi I, et al. In vitro evaluations of topical agents to treat
594   Acanthamoeba keratitis. *Ophthalmology* 2014;121:2059-2065.

595   38.   Alizadeh H, Neelam S, Cavanagh HD. Amoebicidal activities of alexidine against 3
596   pathogenic strains of acanthamoeba. *Eye & contact lens* 2009;35:1-5.

597   39.   Kilvington S, Huang L, Kao E, Powell CH. Development of a new contact lens
598 multipurpose solution: Comparative analysis of microbiological, biological and clinical
599 performance. *J Optom* 2010;3:134–142.
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632 Table 1. Efficacy of topical anaesthetics and fluorescein sodium against *Acanthamoeba* spp.

633 trophozoites and cysts, and toxicity to a human epithelial cell line (Hep2).

	In vitro drug sensitivities (µg/ml)						
	A. castellanii			*A. polyphaga*			Hep2
Drug	MTIC	MTAC	MCC	MTIC	MTAC	MCC	MCT
Proxymetacaine	39	156	156	39	78	156	39
Tetracaine	9.75	19.5	39	19.5	39	78	156
Oxybuprocaine	31.3	250	125	15.6	125	250	125
Lidocaine (+Fluorescein)	312	1250	10000	625	312	1250	5000
Fluorescein sodium (2%)	>10000	>10000	>10000	>10000	>10000	>10000	1250

634 MTIC = minimum trophozoite inhibitory concentration, MTAC = minimum trophozoite

635 amoebicidal concentration (MTAC), MCC = minimum cysticidal concentration, MCT =

636 minimum cytotoxic concentration.

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Table 2. Efficacy of topical antibiotics, antivirals and preservatives against *Acanthamoeba* spp. trophozoites and cysts, and toxicity to a human epithelial cell line (Hep2).

	In vitro drug sensitivities (μg/ml)						
	A. castellanii			*A. polyphaga*			Hep2
Drug	MTIC	MTAC	MCC	MTIC	MTAC	MCC	MCT
[†]**Levofloxacin (Oftaquix®)**	78	156	625	156	312	625	39
Levofloxacin (Pure drug)	312	1250	2500	625	1250	5000	78
Moxifloxacin (Moxeza®)	625	2500	2500	1250	2500	2500	156
Chloramphenicol (Pure drug)	312	625	2500	312	625	1250	625
[†††]**Chloramphenicol** (Generic)	78	312	625	39	156	312	312
Aciclovir (Pure Drug)	63	125	>500	125	250	>500	31.3
[††]**Trifluorothymidine**	312	625	5000	625	1250	2500	156
Benzalkonium Chloride	3.9	7.8	15.6	1	1.95	7.8	31.3
Phenylmercuric Nitrate	1.95	3.9	31.3	1	1.95	15.6	3.9

655 MTIC = minimum trophozoite inhibitory concentration, MTAC = minimum trophozoite

656 amoebicidal concentration, MCC = minimum cysticidal concentration, MCT = minimum

657 cytotoxic concentration. [†] Compound is preserved with benzalkonium chloride (0.005% w/v),

658 [††] compound is preserved with benzalkonium chloride (0.02% w/v), [†††] Compound is

659 preserved with phenylmercuric nitrate (0.002% w/v).

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Table 3. Efficacy of diamidine compounds against *Acanthamoeba* spp. trophozoites and
cysts, and toxicity to a human epithelial cell line (Hep2).

Drug	*In vitro* drug sensitivities (µg/ml)						
	A. castellanii			*A. polyphaga*			Hep2
	MTIC	MTAC	MCC	MTIC	MTAC	MCC	MCT
Propamidine (Pure drug)	62.3	250	>500	250	500	>500	250
†Propamidine (Brolene®)	7.8	15.6	500	15.6	31.3	250	31.3
Hexamidine (Pure drug)	7.8	62.3	250	7.8	31.3	250	62.3
Hexamidine (Desomedine®)	7.8	62.3	250	7.8	31.3	250	62.3
Pentamidine (Pure drug)	62.3	250	>500	125	250	>500	125

MTIC = minimum trophozoite inhibitory concentration, MTAC = minimum trophozoite
amoebicidal concentration, MCC = minimum cysticidal concentration, MCT = minimum
cytotoxic concentration. † Compound is preserved with benzalkonium chloride (0.005% w/v).

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689   Table 4. Efficacy of biguanides and povidone iodine compounds against *Acanthamoeba* spp.

690   for trophozoites and cysts and toxicity to a human epithelial cell line (Hep2).

	In vitro drug sensitivities (µg/ml)						
	A. castellanii			*A. polyphaga*			Hep2
Drug	MTIC	MTAC	MCC	MTIC	MTAC	MCC	MCT
Polyhexamethylene Biguanide	1	3.9	15.6	1	7.8	7.8	31.3
Chlorhexidine	1	3.9	31.3	1.95	15.6	31.3	3.9
Octenidine	1	1.95	7.8	0.5	1	3.9	1.95
Alexidine	1	1.95	3.9	1.95	7.8	7.8	1
Povidone iodine	7.8	31.3	15.6	7.8	31.3	7.8	125

691   MTIC = minimum trophozoite inhibitory concentration, MTAC = minimum trophozoite

692   amoebicidal concentration, MCC = minimum cysticidal concentration, MCT = minimum

693   cytotoxic concentration.

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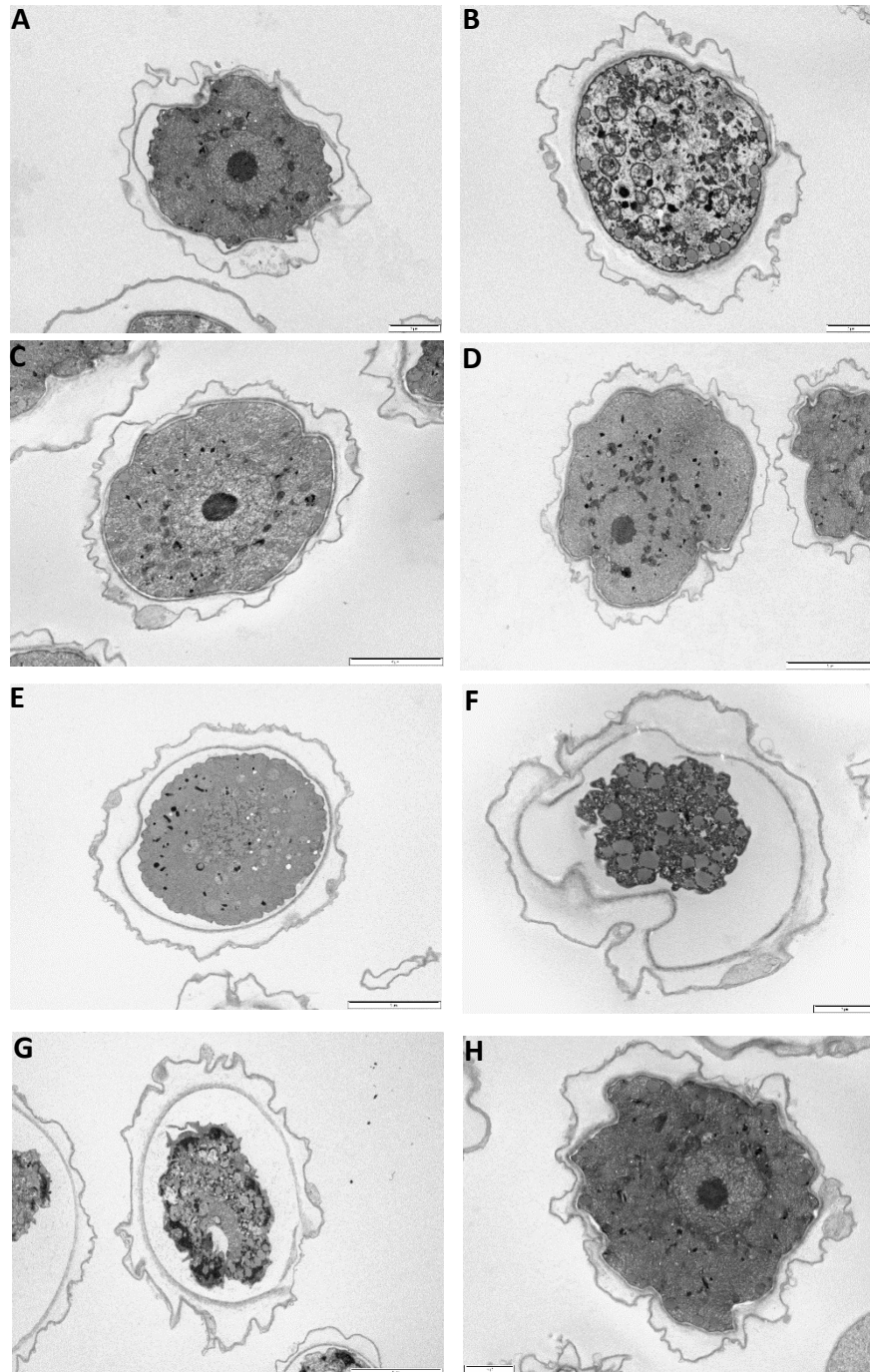
Figure 1: Transmission electron microscopy images of *Acanthamoeba* cyst after 1 hour exposure to the following 7 agents: (A) untreated healthy cyst as control; (B) treated with 1% tetracaine; (C) treated with 0.5% preserved chloramphenicol; (D) treated with 0.1% propamidine pure drug; (E) treated with 0.05 mg/ml benzalkonium chloride; (F) treated with 5% povidone iodine; (G) treated with 0.02% PHMB; (H) treated with 0.5% unpreserved chloramphenicol. Bar = 2 μm.