Free-Living Amoebae Keratitis

Free living amoebae (FLA) keratitis

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Key words: free living amoebae; keratitis; contact lenses; polymerase chain reaction; polyhexamethylene biguanide (PHMB)

ABSTRACT

Purpose: To describe the diagnostic and clinical features and treatment results in 43 consecutive patients with microbiologically proven free-living amoebae (FLA) keratitis.

Methods: In this hospital-based, prospective case series, corneal scrapings from 43 patients with presumed amoebic keratitis were plated on non-nutrient agar. Amoebic isolates were identified morphologically and by polymerase chain reaction (PCR). All patients with culture-proven FLA keratitis were treated with polyhexamethylene biguanide (PHMB) 0.02% eye-drops.

Results: 43 corneal scrapings from 43 patients were found to be culture-positive for FLA; 41 (95%) were from contact lens wearers, 2 (5%) from non-contact lens wearers. Microscopic examination identified 4 *Acanthamoeba* spp, 24 *Hartmannella* spp, 12 vahlkampfiid amoebae, and 3 mixed infections with *Hartmannella*/vahlkampfiid amoebae. Morphological results were confirmed by PCR. Patients with *Acanthamoeba*, *Hartmannella* and vahlkamfiid keratitis had indistinguishable clinical features. In 38 eyes with keratitis at an early stage, treatment with PHMB 0.02% eye-drops was fully successful. In 5 patients with advanced keratitis, topical PHMB 0.02% controlled the infection, but all of them developed a central corneal scar with visual deterioration.

Conclusions: *Acanthamoeba* is not the only cause of amoebic keratitis, because this condition may also be caused by other FLA, such as *Hartmannella* and vahlkampfiid amoebae. This finding is epidemiologically interesting, suggesting a possible different geographical prevalence of the different FLA responsible for keratitis. Early diagnosis and proper anti-amoebic treatment are crucial to yielding a cure.

INTRODUCTION

Pathogenic and opportunistic free-living amoebae (FLA) are aerobic, mitochondriate, eukaryotic protists that occur world-wide and can cause infections in humans and other animals.[1] Amoebic keratitis is a rare, but potentially devastating, corneal infection caused by FLA, such as *Acanthamoeba*, *Hartmannella*, and vahlkampfiid *amoebae*.[2-5] The reported incidence of *Acanthamoeba* keratitis in developed countries is 1-33 cases/1,000,000 contact lens users per year[6] and 1.4 cases/1,000,000 individuals per year in the U.K.[2] In approximately 90% of cases, it occurs in contact lens wearers, accounting for less than 5% of all cases of contact lens-related microbial keratitis.[7,8]

The main risk factors for amoebic keratitis include contact lens use, poor lens hygiene, exposure to contaminated water (tap water, well water, hot-tub water), corneal trauma, use of home-made saline solution, and host susceptibility.[9]

The clinical diagnosis of amoebic keratitis is difficult, because most ophthalmologists are not familiar with this rare corneal infection, which can mimic several types of keratitis (viral, bacterial, or fungal). Laboratory diagnosis of amoebic keratitis is usually based on the direct examination and culture of corneal specimens.[9] FLA can be identified by their morphology, but genus identification requires genetic characterization by polymerase chain reaction (PCR).[7,10-14] Polyhexamethylene biguanide (PHMB) 0.02% has been reported to kill *Acanthamoebae* trophozoites and cysts effectively both in vitro and clinically and, although not licensed for this use, it is presently considered as the gold standard for the treatment of *Acanthamoeba* keratitis.[7]

Little is known about amoebic keratits caused by FLA other than *Acanthamoeba*. The purpose of this study was to describe the diagnostic and clinical features and treatment results in 43 consecutive patients with microbiologically proven FLA keratitis.

MATERIALS AND METHODS

Patients. Forty-three corneal scrapings were obtained from 43 patients with clinically suspected

amoebic keratitis, all examined at the Department of Surgical, Microsurgical, and Medical Sciences, Ophthalmology Unit, University of Sassari, Sassari, Italy, between 2008 and 2015. Amoebic keratitis was suspected on the basis of standard clinical criteria, such as keratitis with punctate epithelial defects and haze, pseudo-dendrites, radial keratoneuritis, limbitis, nummular infiltrates or ring infiltrate in patients wearing contact lenses or after trauma who were not responding to antibiotic or antiviral treatment. Early signs included punctate keratopathy, pseudo-dendrites, radial keratoneuritis, and limbitis, whereas nummular infiltrates and ring keratitis with or without hypopion were considered as late stage signs.[7,9] Taking into consideration the severity of clinical signs, cases were classified as early or late keratitis. All patients with culture-proven FLA keratitis were treated with PHMB 0.02% eye-drops (SIFI, Catania, Italy) with an initial regimen ranging from 6 to 24 times/day, according to the severity of the disease. This product is still investigational. Approval from the local Ethics Committee/Institutional Review Board was obtained and the study was conducted in full accord with the tenets of the Declaration of Helsinki. Each participant received detailed information and provided written informed consent after culture results were available.

Amoebae isolation and culture. Corneal scrapings from patients with presumed amoebic keratitis were plated on non-nutrient agar (NNA) containing 1.5% agar in Page's amoeba saline solution (PAS). The plates were sealed, incubated at 30°C and examined every 24 hours for amoebic growth. Cultures showing the presence of cysts and/or trophozoites were considered positive. These cultures were used to study the morphology and motion characteristics of each isolate and to set up sub-cultures in liquid media. We used two different liquid media: peptone-yeast-glucose (PYG) medium for *Acanthamoeba* cultivation, and peptone-yeast extract-yeast nucleic acid-folic acid-hemin (PYNFH) medium, containing 10% foetal calf serum (FCS), for the cultivation of *Hartmannella*, *Naegleria*, and vahlkampfiid amoebae. When necessary, in order to avoid the risk of co-contamination with other organisms colonizing the ocular surface (e.g., bacteria and fungi), amoebae were axenized by harvesting cysts from the plates and incubating them in 3% HCl

overnight.

Temperature tolerance. Sub-cultures were performed in order to investigate the temperature tolerance of the isolates, a factor correlated with FLA pathogenicity.[15] Parallel cultures were incubated at 30°C, 35°C, and 37°C, respectively, and plates were analyzed daily by phase contrast microscopy.

Phenotypic identification and characterization. Amoebae were identified by phase contrast microscopy, examining the appearance of cysts (size, shape, number of opercula, etc.) and trophozoites. In order to obtain further qualitative information on diversity among the isolates, their type of movement in agar plates and liquid sub-cultures was also evaluated using the classification by Anderson & Rogerson.[16]

Identification by PCR analysis. For molecular biology investigations, amoebae (~$5x10^4$ – $1x10^6$) were harvested from axenic cultures by centrifugation (500 *g* for 10 min). Whole cell DNA was extracted using the QIAamp DNA mini kit (QIAGEN Basel, Switzerland) according to the manufacturer's instructions and eluted in 100 ml AE buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0). Four different previously published PCRs were carried out, using 5 ml of whole cell DNA in a 20-ml reaction volume. *Acanthamoeba*-specific PCR, targeting the ASA.S1 fragment of the 18S rDNA, was performed by using primers JDP1/JDP2 (5'-GGCCCAGATCGTTTACGTGAA-3'/5'-TCTCACAAGCTGCTAGGGGAGTCA-3').[11] The PCR conditions were as follows: 1 cycle of denaturation at 95°C for 5 minutes, followed by 40 repetition cycles at 95°C for 30 seconds, annealing at 55°C for 40 seconds, extension 72°C for 1 minute, and final extension at 72ºC for 1 minute. In order to confirm the presence of FLA DNA in our samples, a second PCR assay, detecting conserved stretches of 18S rDNA, was performed by using primers P-FLA-F/P-FLA-R (5'-CGCGGTAATTCCAGCTCCAATAGC-3'/5'-CAGGTTAAGGTCTCGTTCGTTAAC-3').[12] The following amplification program was used: 1 cycle of denaturation at 95°C for 7 minutes, 40 cycles with denaturation at 95°C for 40 seconds, annealing at 63°C for 30 seconds, primer extension at 72°C for 90 seconds, and final extension at 72°C for 7 minutes. In both PCRs, a clinical strain of

A. castellanii genotype T4, originally isolated from the corneal ulcer of a soft contact lens wearer, served as a reference strain.[17]

A third PCR assay was performed to detect partial 18S rDNA of *Hartmannella* sp. by using primers NA1 (5'-GCTCCAATAGCGTATATTAA-3') and NA2 (5'-AGAAAGAGCTATCAATCTGT-3').[13] Amplification was performed as follows: 1 pre-PCR heat cycle at 94°C for 1 minute; 35 cycles at 94°C for 35 seconds, 50°C for 45 seconds, and 72°C for 1 minute; a final cycle at 72°C for 4 minutes.

For isolates with appearance attributable to the vahlkampfiid amoebae group, internal transcribed spacer-PCR with forward primer ITS1 (5'-GAACCTGCGTAGGGATCATTT-3') and reverse primer ITS2 (5′-TTTCTTTTCCTCCCCTTATTA-3′) was carried out.[14] The following amplification program was used: 1 pre-PCR heat cycle at 94°C for 5 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; a final cycle at 72°C for 5 minutes.

In all PCR assays, sterile saline solution was used as a negative control. Amplification products from all PCRs were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and examined under ultraviolet light.

RESULTS

Forty-three corneal scrapings from 43 patients were found to be culture-positive for FLA; 39 (95%) were from contact lens wearers, 2 (5%) from non-contact lens wearers.

Morphological identification of four isolates revealed *Acanthamoeba* trophozoites with extended pseudopodia or sub-pseudopodia and double wall cysts (Figure 1). Genus identification was later confirmed by *Acanthamoeba*-specific PCR with primers JDP1/JDP2. The PCR products obtained with primers JDP1/JDP2 revealed a 450 bp band (for two strains) and a 550 bp band (for two other strains) on the agarose gel, which are typical of *Acanthamoeba* genotypes T4 and T7, respectively (Figure 2 A). No differences were observed in the growth of these isolates, when they were cultured in PYG or PYNFH medium at 30°C, 35°C, and 37°C.

Twenty-seven FLA isolates showed round, small cysts and worm-shaped trophozoites in liquid cultures, with non-eruptive, cylindrical, monopodial locomotion, such as has been reported for the *Hartmannellidae*.[18,19] On agar plates, trophozoites presented a flat shape without eruptive pseudopod formation (Figure 3). These amoebae showed optimal growth in PYNFH medium with 10% FCS at both 35°C and 37°C. PCR analysis with primers JDP1/JDP2 was negative, whereas PCR products with primers P-FLA-F/P-FLA-R, NA1/NA2, and ITS1/ITS2 yielded an 800 bp band on the agarose gel, which is typical of the *Hartmannella* genus (Figure 2 B, C, D).

Fifteen FLA isolates showed round to slightly oval cysts with smooth walls. Trophozoites were pleiomorphic with a large, oval nucleus with centrally located dark chromatin. They presented a single, large pseudopodium or ectoplasmic outbursts of different size with a hyaline-appearing cytoplasm and eruptive locomotion, as has been reported for *Vahlkampfia* and *Naegleria* spp.[20] All these isolates grew only in T25 flasks pre-coated with NNA containing PYNFH medium with 10% FCS at both 35°C and 37°C. To exclude the presence of flagellate amoebae, such as *Naegleria* spp., trophozoites growing on PYNFH monolayers were washed with phosphate buffered saline solution, transferred into separate flasks containing distilled water, and incubated at 37°C. Upon suspension in distilled water, the isolates did not transform into flagellates, even after 48 hours' exposure. PCR analysis with both primers P-FLA-F/P-FLA-R and NA1/NA2 did not yield amplification products, whereas PCR with primers ITS1/ITS2 produced a single fragment of approximately 600 bp, which is typical of vahlkampfiid amoebae (Fig. 2 B, C, D).

Overall, laboratory analysis identified 4 (9.3%) *Acanthamoeba* spp, 24 (55.8%) *Hartmannella* spp, 12 (27.9%) vahlkampfiid amoebae, and 3 (7%) mixed infections with *Hartmannella*/vahlkampfiid amoebae.

In the two non-contact lens wearers, amoebic keratitis was caused by *Hartmannella* spp. Patients with *Acanthamoeba*, *Hartmannella* and/or vahlkamfiid amoebae keratitis had indistinguishable clinical features (Table 1).

In 38 eyes with FLA keratitis at an early stage, treatment with PHMB 0.02% eye-drops, given for a

minimum of three months, was fully successful. In 5 patients with advanced keratitis, topical PHMB 0.02% for an average of 7 months controlled the infection, but all of them developed a central corneal scar with visual deterioration, which required corneal graft (Fig. 4). There was no difference in treatment outcome of cases caused by each of the different FLA.

Treatment with PHMB 0.02% eye-drops was generally well-tolerated and no patients had systemic side effects; eye burning sensation and/or itching were reported in all the patients under intensive treatment (> 8times/day).

DISCUSSION

Acanthamoeba keratitis is a severe, often sight-threatening, corneal infection, which is challenging to diagnose and manage.[7] Other genera of FLA, such as *Hartmannella*, and *Vahlkampfiia*, have been isolated from patients with contact-lens related keratitis;[4,5,21,22] hence, *Acanthamoeba* is not the only organism causing amoebic keratitis. Even though *Hartmannella* has recently been included in a list of human parasites,[23] the ability of *Hartmannella* and *Vahlkampfiia* to produce disease in humans is still controversial.[24] Nevertheless, experimental evidence indicates that the last two genera of FLA produce a cytopathic effect to human keratocytes similar to that produced by *Acanthamoeba*. This result supports the idea that both *Hartmannella* and *Vahlkampfiia* can produce opportunistic corneal infection.[25]

In our study, all the patients with clinically suspected ameobic keratitis were found to be culture positive for FLA. All the isolates were able to grow at 35°C and 37°C. This is an interesting finding, because temperature tolerance is a factor correlated with FLA pathogenicity and may be a good indicator of the potential virulence of a given isolate.[15]

On slit-lamp examination, *Acanthamoeba*, *Hartmannella* and/or vahlkamfiid amoebae keratitis presented with indistinguishable clinical features. In the early stages, conjunctival hyperaemia, punctate keratopathy, epithelial haze, pseudodendrites, radial keratoneuritis, and limbitis were observed in different combinations. All five patients with FLA keratitis at an advanced stage

showed a ring infiltrate with hypopion. Interestingly, this condition was caused by either *Acanthamoeba* or *Hartmannella*/vahlkampfiid amoebae co-infection. The exact meaning of this finding is unclear; possibly, some strains of *Acanthamoeba* and mixed infection with *Hartmannella* and vahlkampfiid amoebae may be more virulent and cause more severe keratitis than *Hartmannella* and vahlkampfiid amoebae alone.

Culture of corneal specimens is the gold standard for the laboratory diagnosis of amoebic keratitis, but today several PCR-based techniques with high sensitivity (up to 85%) have become available.[7,9] An important clinical limitation of PCR is that it cannot differentiate between live and dead organisms, so cannot indicate whether the infection is still active or the cornea is sterile.

A positive culture is essential for the morphological identification of FLA; however, morphology alone may sometimes not be sufficient.[1] In such cases, FLA identification at the genus level can be achieved by PCR methods. [7,10-14] PCR with primers P-FLA-F and P-FLA-R, targeting conserved sequences of the 18S rDNA gene, can be used to confirm the presence of FLA DNA and its quality.[12] This PCR produces amplicons of ~800 bp in the presence of *Hartmannella* DNA and ~1080 bp in the presence of *Acanthamoeba* DNA.[12] PCR with primers JDP1 and JDP2, targeting the ASA.S1 fragment of the 18S rDNA, is highly specific for the genus *Acanthamoeba*.[11] Similarly, PCR with primers NA1 and NA2, detecting partial 18S rDNA of *Hartmannella*, is highly specific for this genus.[13] On the other hand, isolates belonging to the family *Vahlkampfiidae* can be identified by sequencing the PCR-amplified ITS1, 5.8S, and ITS2 rDNA.[14] Even though PCR has become increasingly popular for an easier discrimination of the different FLA species and genera and for studying their genetic relationship, knowledge of morphology is still required when identifying amoebic isolates by molecular techniques.[19] A potential limitation of our study is that the type of PCR performed was based on the morphologic identification of FLA. However, this selective approach, essential to confirm morphology results, is much less expensive than performing all PCR types on all positive cultures.

Unlike former reports on amoebic keratitis, which found a predominance of *Acanthamoeba*

genotype T4,[10,11,26] our study on FLA isolated from corneal specimens of patients from the island of Sardinia, Italy, showed the predominance of the genera *Hartmannella* and vahlkampfiid amoebae. As far as we are aware, this is the largest series of cases of keratitis caused by *Hartmannella* and vahlkampfiid amoebae. This finding is epidemiologically interesting, suggesting a possible different geographical prevalence of the various FLA responsible for keratitis. At present, the genera *Hartmannella* and *Vahlkampfiia*, in contrast to *Acanthamoeba*, are not subject to specific laboratory handling precautions. Evidence that they are potential pathogens would necessitate modification of this practice while dealing with samples from patients with presumed amoebic keratitis.

In-vitro experiments have demonstrated that anti-amoebic agents, such as PHMB, chlorhexidine and propamidine isethionate, which are active against *Acanthamoeba*, are also effective against corneal isolates of *Hartmannella* and amoebae belonging to the family *Vahlkampfiidae*.[4] However, some strains have been reported to be resistant to propamidine isethionate,[27] thus this drug is generally not recommended for monotherapy. In our study, PHMB 0.02% eye-drops yielded complete resolution of FLA keratitis, when the infection was diagnosed at an early stage. In the cases with advanced keratitis, the inflammation subsided gradually with topical PHMB 0.02% given for 6-8 months. After treatment, the affected eyes were quiet and there was central corneal scarring, which required penetrating keratoplasty.

In conclusion, our study confirmed that *Acanthamoeba* is not the only cause of amoebic keratitis, because this condition may also be due to other FLA, such as *Hartmannella* and amoebae belonging to the family *Vahlkampfiidae*. Therefore, FLA other than *Acanthamoeba* should be considered in the diagnosis of presumed amoebic keratitis, when *Acanthamoeba* cannot readily be cultured or identified. Both morphology and PCR-based methods are essential to identify correctly the different genera of FLA. Patients with *Acanthamoeba*, *Hartmannella*, and *vahlkamfiid* keratitis showed indistinguishable clinical features. Irrespective of the amoebic species causing keratitis, early diagnosis and proper anti-amoebic treatment are crucial to yielding a cure. Further experimental and clinical studies are necessary to support the idea that both *Hartmannella* and amoebae belonging to

261 the family *Vahlkampfiidae* are potential corneal pathogens and for a better understanding of the
262 mechanism by which FLA may cause keratitis.
263
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271 and Eye Research (EVER), Nice (France) 5th-8th October 2016.
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REFERENCES

1. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol Med Microbiol* 2007;50:1–26

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

3. Schaumberg DA, Snow KK, Dana MR. The epidemic of *Acanthamoeba* keratitis: where do we stand? *Cornea* 1998;17:3–10.

4. Aitken D, Hay J, Kinnear FB et al. Amebic keratitis in a wearer of disposable contact lenses due to a mixed *Vahlkampfia* and *Hartmannella* infection. *Ophthalmology* 1996;103:485–494.

5. Inoue T, Asari S, Tahara K et al. *Acanthamoeba* keratitis with symbiosis of *Hartmannella* ameba. *Am J Ophthalmol* 1998;125:721–723.

6. Centers for Disease Control and Prevention. *Acanthamoeba* Infection – Epidemiology & Risk Factors [CDC web site]. August 24, 2012. Available at: https://www.cdc.gov/parasites/acanthamoeba/epi.html. Accessed March 1, 2017

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

8. Sharma S, Garg P, Rao GN. Patient characteristics, diagnosis, and treatment of non-contact lens related *Acanthamoeba* keratitis. *Br J Ophthalmol* 2000;84:1103–1108.

9. Carnt N, Stapleton F. Strategies for the prevention of contact lens-related *Acanthamoeba* keratitis: a review. *Ophthalmic Physiol Opt* 2016;36:77–92.

10. Yera H, Zamfir O, Bourcier T et al. The genotypic characterization of *Acanthamoeba* isolates from human ocular samples. *Br J Ophthalmol* 2008;92:1139–1141.

11. Schroeder JM, Booton GC, Hay J et al. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. *J Clin Microbiol* 2001;39:1903–1911.

313 12. Tsvetkova N, Schild M, Panaiotov S et al. The identification of free-living environmental
314 isolates of amoebae from Bulgaria. *Parasitol Res* 2004;92:405–413.
315 13. Lasjerdi Z, Niyyati M, Haghighi A et al. Potentially pathogenic free-living amoebae isolated
316 from hospital wards with immunodeficient patients in Tehran, Iran. *Parasitol Res* 2011;109:575–
317 580.
318 14. De Jonckheere JF, Brown S. The identification of vahlkampfiid amoebae by ITS sequencing.
319 *Protist* 2005;156:89–96.
320 15. Rohr U, Weber S, Michel R et al. Comparison of free-living amoebae in hot water systems of
321 hospitals with isolates from moist sanitary areas by identifying genera and determining temperature
322 tolerance. *Appl Environ Microbiol* 1998;64:1822–1824.
323 16. Anderson OR, Rogerson A. Annual abundances and growth-potential of gymnamoebae in the
324 Hudson estuary with comparative data from the Firth of Clyde. *Eur J Protistol* 1995;31:223–233.
325 17. Zanetti S, Fiori PL, Pinna A et al. Susceptibility of *Acanthamoeba castellanii* to contact lens
326 disinfecting solutions. Antimicrob Agents Chemother 1995;39:1596–1598.
327 18. Page FC. Taxonomic criteria for limax amoebae, with descriptions of 3 new species of
328 *Hartmannella* and 3 of *Vahlkampfia*. *J Protozool* 1967;14:499–521.
329 19. De Jonckheere JF, Gryseels S, Eddyani M. Knowledge of morphology is still required when
330 identifying new amoeba isolates by molecular techniques. *Eur J Protistol* 2012;48:178–184.
331 20. González-Robles A, Salazar-Villatoro L, González-Lázaro M et al. *Vahlkampfia* sp: Structural
332 observations of cultured trophozoites. *Exp Parassitol* 2012;130:86–90.
333 21. Alexandrakis G, Miller D, Huang A. Amebic keratitis due to *Vahlkampfia* infection following
334 corneal trauma. *Arch Ophthalmol* 1998;116:950–951.
335 22. Dua HS, Azuara-Blanco A, Hossain M et al. Non *Acanthamoeba* amebic keratitis. *Cornea*
336 1998;17:675–677.
337 23. Garcia LS. Classification of human parasites. *Clin Infect Dis* 1997; 25:21–23.
338 24. De Jonckheere JF, Brown S. Non-*Acanthamoeba* amoebic infection. *J Infect* 1998;36:349–50.

339 25. Kinnear FB. Cytopathogenicity of *Acanthamoeba*, *Vahlkampfia* and *Hartmannella*: quantative
340 & qualitative in vitro studies on keratocytes. *J Infect* 2003;46:228–237.
341 26. Booton GC, Kelly DJ, Chu YW et al. 18S ribosomal DNA typing and tracking of
342 *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home
343 water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *J Clin Microbiol* 2002;40:1621–
344 1625.
345 27. Kilvington S, Hughes R, Byas J et al. Activities of therapeutic agents and myristamidopropyl
346 dimethylamine against *Acanthamoeba* isolates. *Antimicrob Agents Chemother* 2002;46:2007–2009.
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365 **Figure legends**

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367 **Figure 1.** Phase-contrast microscopy images of three clinical isolates from patients with suspected
368 amoebic keratitis, included in the *Acanthamoeba* genus. Cysts and trophozoites of isolate IA1 (**A,**
369 **B**) and isolate IA2 (**C, D)**; cyst of isolate IA3 (**E**), all observed in PYG medium. Original
370 magnification, x 600 (cysts); x 400 (trophozoites).

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372 **Figure 2.** Agarose gel electrophoresis of DNA amplification of various free-living amoebae isolates
373 obtained by polymerase chain reaction with JDP primers only **(A)**, both JDP and FLA primers **(B)**,
374 NA primers **(C)**, and ITS primers **(D)**. **A:** PCR with JDP primers, specific for the *Acanthamoeba*
375 genus. Clinical isolates IA1 and IA2, as well as the positive control (*A. castellanii* genotype T4),
376 yielded single bands of 450 bp, whereas isolate IA3 produced a band of 550 bp. **B:** PCR with FLA
377 primers, generic for free-living amoebae. Isolates morphologically included in the *Hartmannella*
378 genus, such as IH1, and *Acanthamoeba* genus, such as IA1, yielded bands of 800 bp and 1078 bp,
379 respectively, but no bands were observed with isolates included in the vahlkamfiid amoebae group.
380 **C:** PCR with NA primers, specific for the *Hartmannella* genus. Some isolates, such as strain IH1,
381 morphologically included in the *Hartmannella* genus, produced a band of 800 bp. **D:** ITS PCR
382 produced single fragments of approximately 600 and 800 bp with isolates classified into the
383 vahlkampfiid amoebae or *Hartmannella* genus, respectively. M, molecular markers (100-bp DNA
384 ladder).

385

386 **Figure 3.** Phase-contrast microscopy images of corneal isolate IH1, included in the *Hartmannella*
387 genus. Both the cysts and trophozoite are on NNA plates (original magnification, x 600 and x 400,
388 respectively).

389

390 **Figure 4.** Large, central corneal scar in a patient with initially severe contact lens-related keratitis

(ring infiltrate with hypopion) caused by mixed infection with *Hartmannella*/vahlkampfiid amoebae. Topical PHMB 0.02% for 6 months controlled the infection, thus allowing a successful corneal graft.

Figure 1.

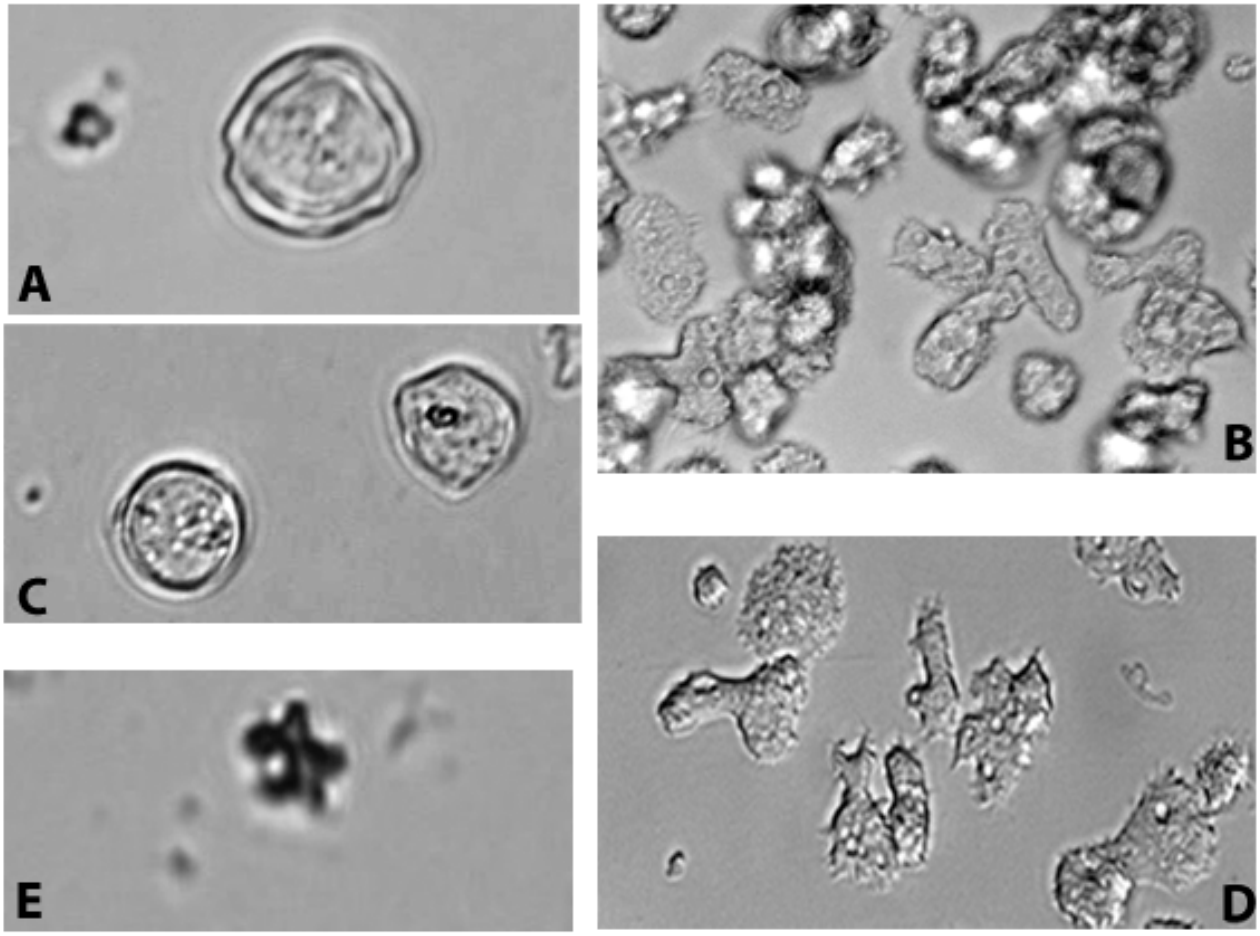
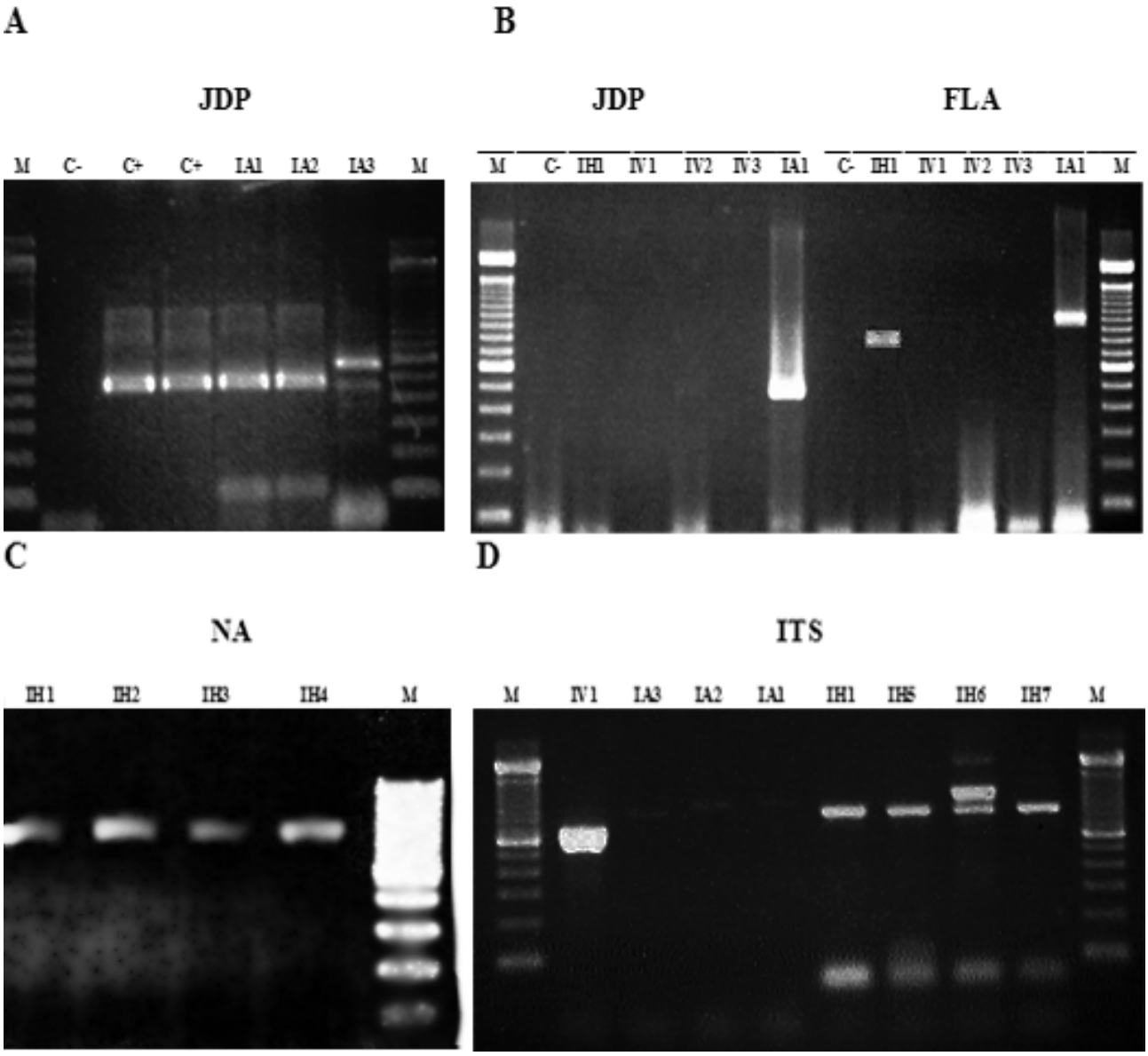


Figure 2.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JDP
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C- IH1 IV1 IV2 IV3 IA1 M
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IH1 IH2 IH3 IH4 M
D
ITS
M IV1 IA3 IA2 IA1 IH1 IH5 IH6 IH7 M

Figure 3.

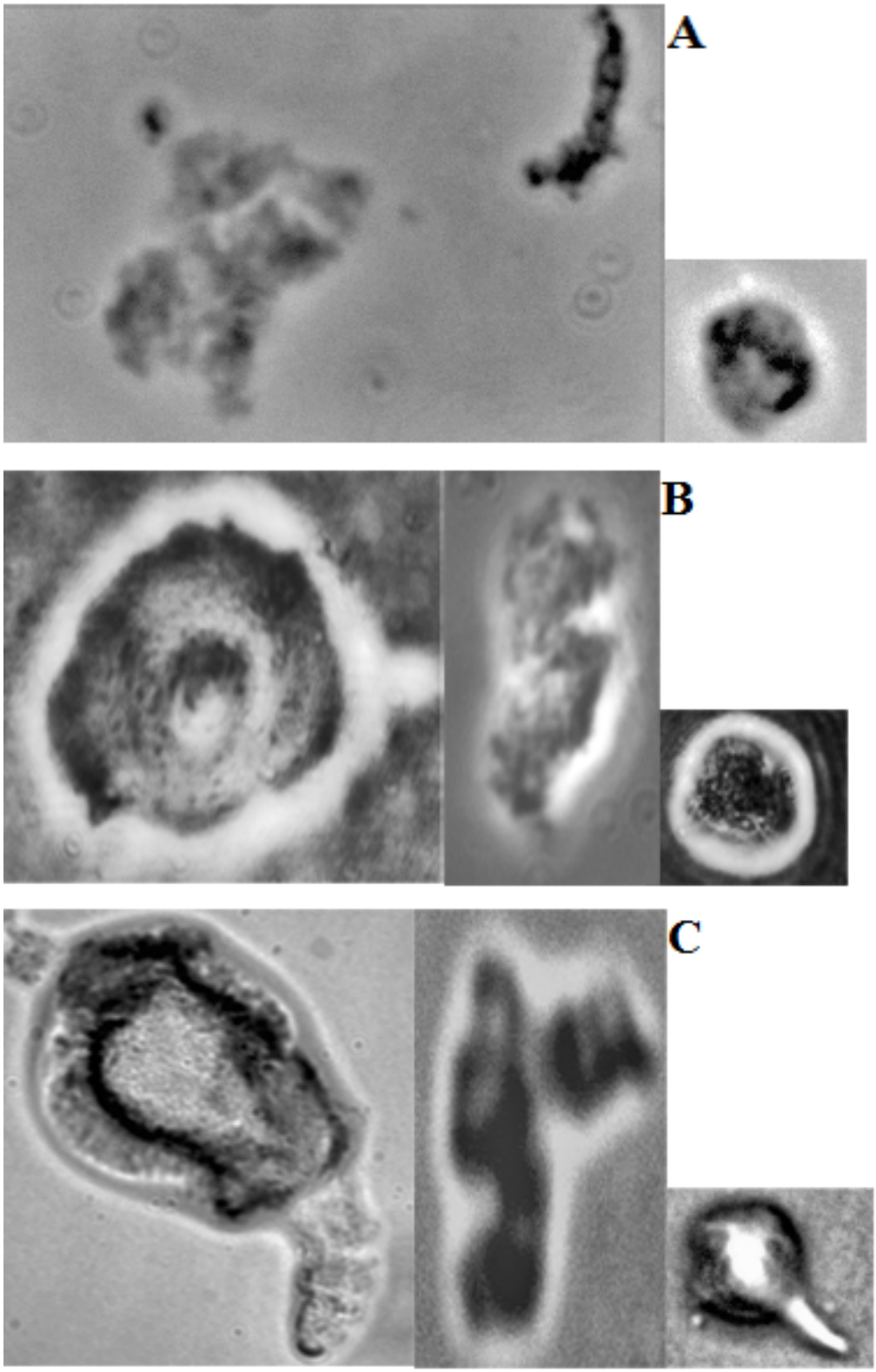

Figure 4.