

Free-Living Amoebae Keratitis

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1 **Free living amoebae (FLA) keratitis**

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23

24 **Key words:** free living amoebae; keratitis; contact lenses; polymerase chain reaction;

25 polyhexamethylene biguanide (PHMB)

26

27 **ABSTRACT**

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

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

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

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

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53 INTRODUCTION

54 Pathogenic and opportunistic free-living amoebae (FLA) are aerobic, mitochondriate, eukaryotic
55 protists that occur world-wide and can cause infections in humans and other animals.¹ Amoebic
56 keratitis is a rare, but potentially devastating, corneal infection caused by FLA, such as
57 *Acanthamoeba*, *Hartmannella*, and vahlkampfiid *amoebae*.²⁻⁵ The reported incidence of
58 *Acanthamoeba* keratitis in developed countries is 1-33 cases/1,000,000 contact lens users per year⁶
59 and 1.4 cases/1,000,000 individuals per year in the U.K.² In approximately 90% of cases, it occurs
60 in contact lens wearers, accounting for less than 5% of all cases of contact lens-related microbial
61 keratitis.^{7,8}

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

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

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

76

77 MATERIALS AND METHODS

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

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

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

105 overnight.

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

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

115 **Identification by PCR analysis.** For molecular biology investigations, amoebae ($\sim 5 \times 10^4 - 1 \times 10^6$)
116 were harvested from axenic cultures by centrifugation (500 g for 10 min). Whole cell DNA was
117 extracted using the QIAamp DNA mini kit (QIAGEN Basel, Switzerland) according to the
118 manufacturer's instructions and eluted in 100 µl AE buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH
119 9.0). Four different previously published PCRs were carried out, using 5 µl of whole cell DNA in a
120 20-µl reaction volume. *Acanthamoeba*-specific PCR, targeting the ASA.S1 fragment of the 18S
121 rDNA, was performed by using primers JDP1/JDP2 (5'-GGCCCAGATCGTTTACGTGAA-3'/5'-
122 TCTCACAAGCTGCTAGGGGAGTCA-3').¹¹ The PCR conditions were as follows: 1 cycle of
123 denaturation at 95°C for 5 minutes, followed by 40 repetition cycles at 95°C for 30 seconds,
124 annealing at 55°C for 40 seconds, extension 72°C for 1 minute, and final extension at 72°C for 1
125 minute. In order to confirm the presence of FLA DNA in our samples, a second PCR assay,
126 detecting conserved stretches of 18S rDNA, was performed by using primers P-FLA-F/P-FLA-R
127 (5'-CGCGGTAATTCCAGCTCCAATAGC-3'/5'-CAGGTAAAGGTCTCGTTCGTTAAC-3').¹²
128 The following amplification program was used: 1 cycle of denaturation at 95°C for 7 minutes, 40
129 cycles with denaturation at 95°C for 40 seconds, annealing at 63°C for 30 seconds, primer extension
130 at 72°C for 90 seconds, and final extension at 72°C for 7 minutes. In both PCRs, a clinical strain of

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

133 A third PCR assay was performed to detect partial 18S rDNA of *Hartmannella* sp. by using primers
134 NA1 (5'-GCTCCAATAGCGTATATTAA-3') and NA2 (5'-AGAAAGAGCTATCAATCTGT-
135 3').¹³ Amplification was performed as follows: 1 pre-PCR heat cycle at 94°C for 1 minute; 35
136 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
137 4 minutes.

138 For isolates with appearance attributable to the vahlkampfiid amoebae group, internal transcribed
139 spacer-PCR with forward primer ITS1 (5'-GAACCTGCGTAGGGATCATTT-3') and reverse
140 primer ITS2 (5'-TTTCTTTTCCTCCCCTTATTA-3') was carried out.¹⁴ The following
141 amplification program was used: 1 pre-PCR heat cycle at 94°C for 5 minutes; 35 cycles at 94°C for
142 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; a final cycle at 72°C for 5 minutes.

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

146

147 **RESULTS**

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

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

157 Twenty-seven FLA isolates showed round, small cysts and worm-shaped trophozoites in liquid
158 cultures, with non-eruptive, cylindrical, monopodial locomotion, such as has been reported for the
159 *Hartmannellidae*.^{18,19} On agar plates, trophozoites presented a flat shape without eruptive
160 pseudopod formation (Figure 3). These amoebae showed optimal growth in PYNFH medium with
161 10% FCS at both 35°C and 37°C. PCR analysis with primers JDP1/JDP2 was negative, whereas
162 PCR products with primers P-FLA-F/P-FLA-R, NA1/NA2, and ITS1/ITS2 yielded an 800 bp band
163 on the agarose gel, which is typical of the *Hartmannella* genus (Figure 2 B, C, D).

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

176 Overall, laboratory analysis identified 4 (9.3%) *Acanthamoeba* spp, 24 (55.8%) *Hartmannella* spp,
177 12 (27.9%) vahlkampfiid amoebae, and 3 (7%) mixed infections with *Hartmannella*/vahlkampfiid
178 amoebae.

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

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

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

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

190

191 **DISCUSSION**

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

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

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

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

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

218 A positive culture is essential for the morphological identification of FLA; however, morphology
219 alone may sometimes not be sufficient.¹ In such cases, FLA identification at the genus level can be
220 achieved by PCR methods.^{7,10-14} PCR with primers P-FLA-F and P-FLA-R, targeting conserved
221 sequences of the 18S rDNA gene, can be used to confirm the presence of FLA DNA and its
222 quality.¹² This PCR produces amplicons of ~800 bp in the presence of *Hartmannella* DNA and
223 ~1080 bp in the presence of *Acanthamoeba* DNA.¹² PCR with primers JDP1 and JDP2, targeting
224 the ASA.S1 fragment of the 18S rDNA, is highly specific for the genus *Acanthamoeba*.¹¹ Similarly,
225 PCR with primers NA1 and NA2, detecting partial 18S rDNA of *Hartmannella*, is highly specific
226 for this genus.¹³ On the other hand, isolates belonging to the family *Vahlkampfiidae* can be
227 identified by sequencing the PCR-amplified ITS1, 5.8S, and ITS2 rDNA.¹⁴ Even though PCR has
228 become increasingly popular for an easier discrimination of the different FLA species and genera
229 and for studying their genetic relationship, knowledge of morphology is still required when
230 identifying amoebic isolates by molecular techniques.¹⁹ A potential limitation of our study is that
231 the type of PCR performed was based on the morphologic identification of FLA. However, this
232 selective approach, essential to confirm morphology results, is much less expensive than performing
233 all PCR types on all positive cultures.

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

235 genotype T4,^{10,11,26} our study on FLA isolated from corneal specimens of patients from the island of
236 Sardinia, Italy, showed the predominance of the genera *Hartmannella* and vahlkampfiid amoebae.
237 As far as we are aware, this is the largest series of cases of keratitis caused by *Hartmannella* and
238 vahlkampfiid amoebae. This finding is epidemiologically interesting, suggesting a possible different
239 geographical prevalence of the various FLA responsible for keratitis. At present, the genera
240 *Hartmannella* and *Vahlkampfiia*, in contrast to *Acanthamoeba*, are not subject to specific laboratory
241 handling precautions. Evidence that they are potential pathogens would necessitate modification of
242 this practice while dealing with samples from patients with presumed amoebic keratitis.
243 *In-vitro* experiments have demonstrated that anti-amoebic agents, such as PHMB, chlorhexidine
244 and propamidine isethionate, which are active against *Acanthamoeba*, are also effective against
245 corneal isolates of *Hartmannella* and amoebae belonging to the family *Vahlkampfiidae*.⁴ However,
246 some strains have been reported to be resistant to propamidine isethionate,²⁷ thus this drug is
247 generally not recommended for monotherapy. In our study, PHMB 0.02% eye-drops yielded
248 complete resolution of FLA keratitis, when the infection was diagnosed at an early stage. In the
249 cases with advanced keratitis, the inflammation subsided gradually with topical PHMB 0.02% given
250 for 6-8 months. After treatment, the affected eyes were quiet and there was central corneal scarring,
251 which required penetrating keratoplasty.

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

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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).

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

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

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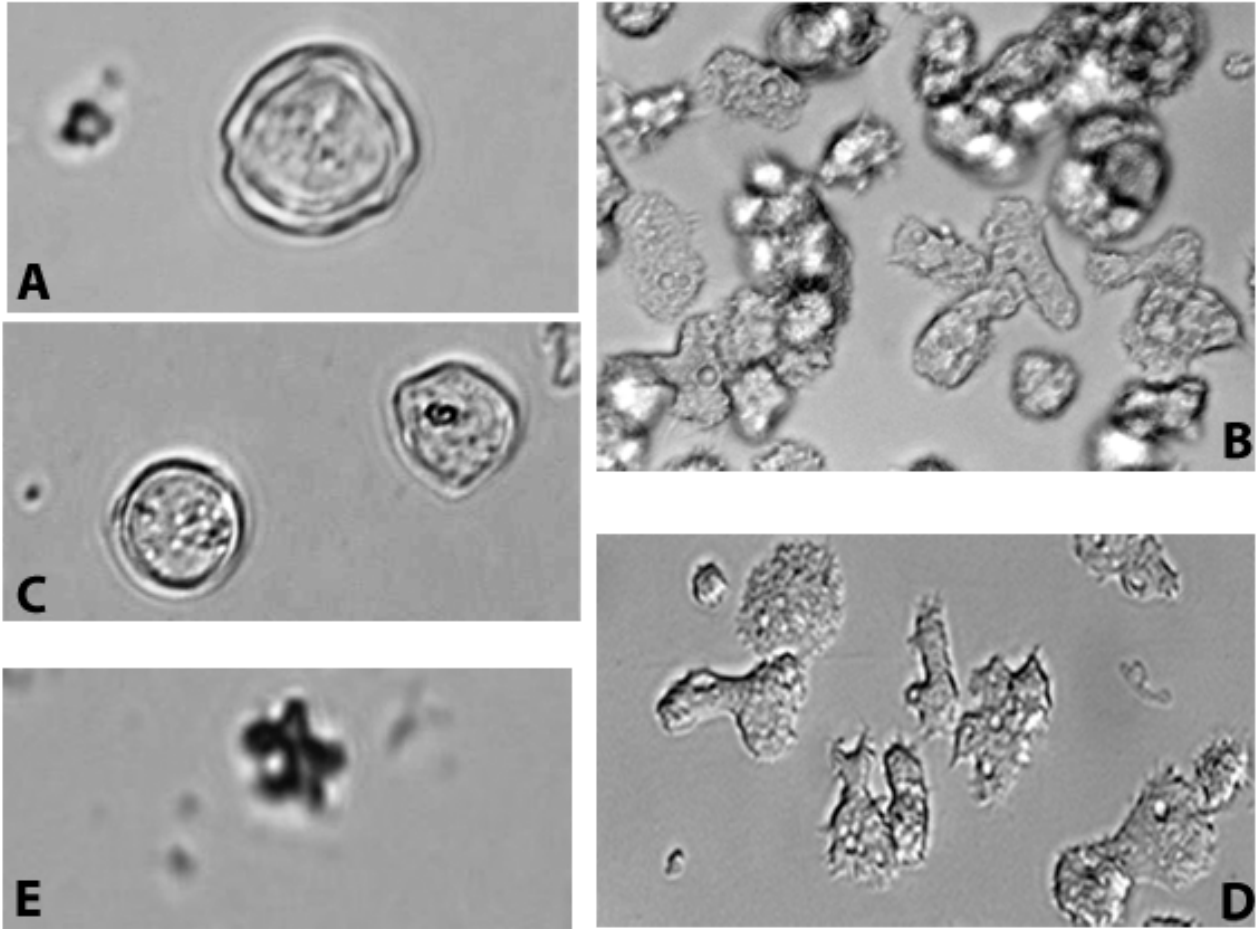
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417 **Figure 1.**



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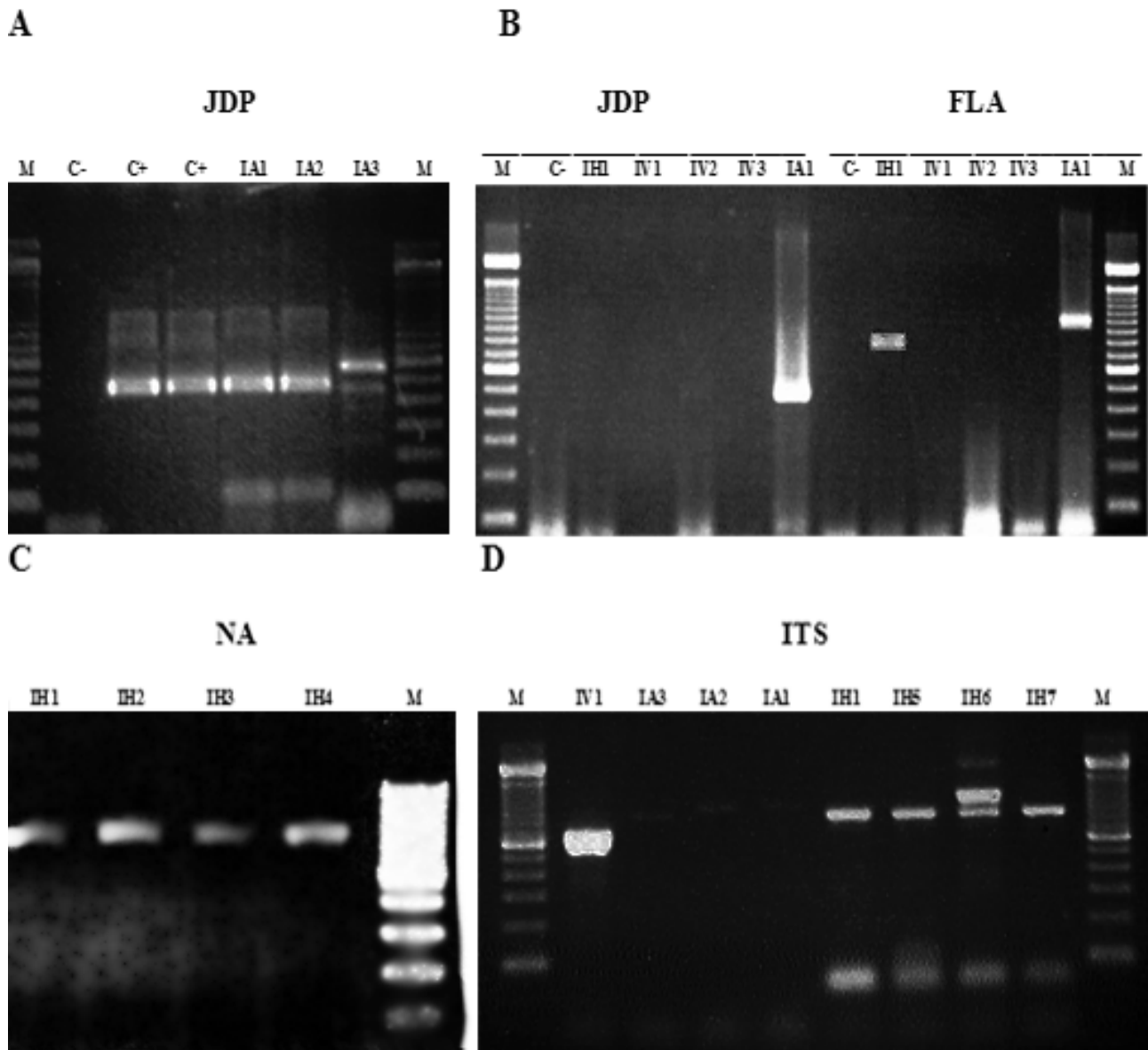
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430 **Figure 2.**

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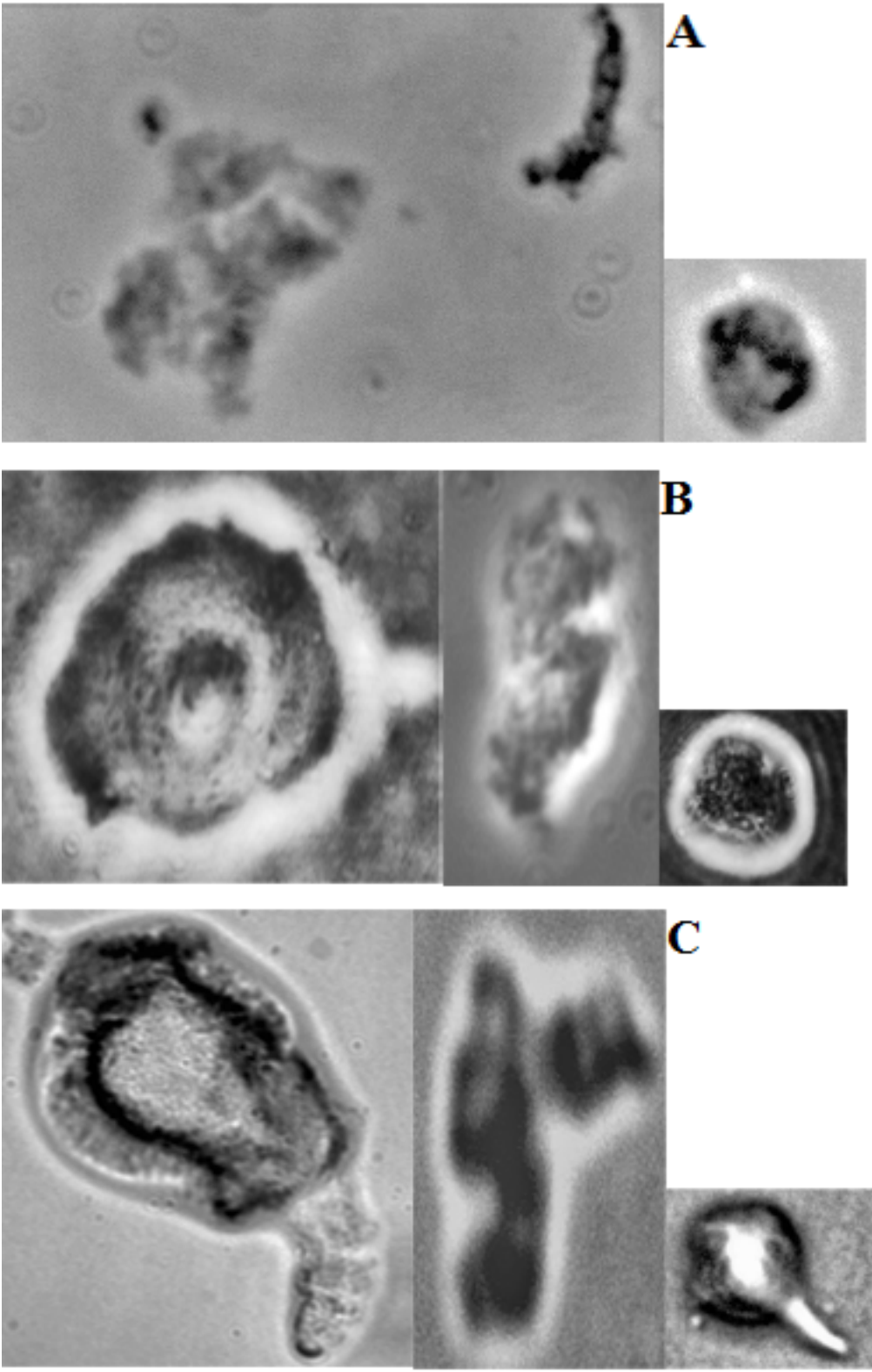
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440 **Figure 3.**



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445 **Figure 4.**



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