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1 Prospective study of the diagnostic accuracy of the *In Vivo* Laser Scanning Confocal
2 Microscope for Severe Microbial Keratitis

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16

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26 bacteria, fungus, acanthamoeba

27

Precis

The HRT3 *in vivo* confocal microscope has a high sensitivity and specificity for detection of fungi and acanthamoeba, with good inter and intragrader agreement, and superior organism detection in deep ulcers compared to standard microbiology.

28 **Abstract (345 Words)**

29

30 **Objective:** To determine the diagnostic accuracy of *in vivo* confocal microscopy
31 (IVCM) for moderate to severe microbial keratitis (MK).

32

33 **Design:** Double-masked prospective cohort study.

34

35 **Study participants:** Consecutive patients presenting to Aravind Eye Hospital,
36 Madurai, India between Feb 2012 and Feb 2013 with MK (diameter ≥ 3 mm, excluding
37 descemetocele, perforation or herpetic keratitis).

38

39 **Methods:** Following examination, the corneal ulcer was scanned by IVCM
40 (HRT3/RCM, Heidelberg Engineering). Images were graded for presence/absence of
41 fungal hyphae or acanthamoeba cysts by the confocal microscopist who performed
42 the scan (masked to microbial diagnosis) and four other experienced confocal graders
43 (masked to clinical features and microbiology). Regrading of shuffled image set was
44 performed by 3 graders, 3 weeks later. Corneal scrape samples were collected for
45 microscopy and culture.

46

47 **Main Outcome Measures:** Sensitivity, specificity, positive and negative predictive
48 values of IVCM compared to reference standard of positive culture and/or light
49 microscopy. Sensitivities and specificities for multiple graders were pooled and 95%
50 confidence intervals calculated using a bivariate random-effects regression model.

51

52 **Results:** 239 patients with MK were enrolled. Fungal infection was detected in 176
53 (74%) and acanthamoeba in 17 (7%) by microbiology. IVCN had an overall pooled
54 (5 graders) sensitivity of 85.7% (95% CI 82.2% - 88.6%) and pooled specificity of
55 81.4% (95% CI 76.0% - 85.9%) for fungal filament detection. For acanthamoeba, the
56 pooled sensitivity was 88.2% (95% CI 76.2% - 94.6%) and pooled specificity was
57 98.2% (95% CI 94.9% - 99.3%). Inter-grader agreement was good: kappa=0.88 for
58 definite fungus, kappa=0.72 for definite acanthamoeba. Intra-grader repeatability was
59 high for both definite fungus (kappa 0.88 - 0.95) and definite acanthamoeba
60 classification (kappa 0.63 - 0.90). IVCN images from eleven patients were
61 considered by all five graders to have a specific organism present (ten fungus, one
62 acanthamoeba) but were culture and light microscopy negative.

63

64 **Conclusions:** Laser scanning IVCN performed with experienced confocal graders
65 has a high sensitivity, specificity and test reproducibility for detecting fungal
66 filaments and acanthamoeba cysts in moderate to large corneal ulcers in India. This
67 imaging modality was particularly useful for detecting organisms in deep ulcers in
68 which culture and light microscopy were negative.

69

70 **Introduction**

71 Severe microbial keratitis (MK) is an important cause of blindness worldwide.¹ In
72 recent years, outbreaks of fungal and acanthamoeba keratitis have brought to light the
73 complexity of identifying a causative organism in these infections.² Although
74 experienced cornea specialists can correctly identify fungal from bacterial keratitis
75 based on clinical features alone in up to 66% of cases,³ larger ulcers can present a
76 diagnostic challenge as tissue destruction can obscure classical features.² In these
77 cases, microbiological techniques such as culture and light microscopy can aid in
78 diagnosis but they do not offer a high diagnostic accuracy. Culture positivity rates in
79 microbial keratitis vary widely from 40 to 73% in different settings, most likely due to
80 the small size of corneal scrape samples, prior antimicrobial treatment inhibiting
81 microbial growth, and the fastidious nature of some organisms requiring special
82 growth media (e.g. fungi and acanthamoeba).⁴⁻⁷ Direct visualization of fungal
83 filaments or acanthamoeba cysts in corneal scrapings using light microscopy can give
84 a higher detection rate when compared to culture alone,⁸ but relies upon availability
85 of trained, experienced observers who may not be present in some healthcare settings.

86

87 *In vivo* confocal microscopy (IVCM) is a non-invasive imaging technique that allows
88 direct visualization of pathogens within the patient's cornea.⁹ The two imaging
89 modalities in current clinical use are the scanning slit IVCM (Confoscan, Nidek
90 Technologies, Fremont, CA) and the laser scanning IVCM (HRT3 with Rostock
91 Corneal Module, RCM, Heidelberg Engineering, Germany). The confoscan has a
92 resolution of 1 micron laterally and up to 24 microns axially; the HRT3/RCM also has
93 a lateral resolution of 1 micron but higher axial resolution of 7.6 microns.¹⁰ Although
94 many have reported the ability of both of these confocal microscopes to detect fungal

95 filaments and acanthamoeba cysts in human microbial keratitis *in vivo* (summarized
96 in Labbe *et al*⁹), only two studies have prospectively assessed the diagnostic accuracy
97 of IVCN compared to standard microbiological techniques of culture with or without
98 light microscopy.^{11,12} Kanavi *et al* found that with a single IVCN grader the
99 Confoscan 3.0 IVCN had a sensitivity of 100% for detection of acanthamoeba and
100 specificity of 84%, compared to culture as the reference standard. For fungal
101 filaments, the sensitivity was also high (94%) with a lower specificity (78%). The
102 authors do not state whether the IVCN grader was masked to data from clinical
103 assessment of the patient. Vaddavalli *et al* also used the Confoscan 3.0, with two
104 IVCN graders who were masked to both the microbiological diagnosis and clinical
105 assessment.¹² They found a sensitivity of 80% and specificity of 100% for the
106 detection of acanthamoeba cysts. For fungal filament detection they found a
107 sensitivity of 89.2% and specificity of 92.7%. In addition, a good inter-observer
108 agreement (kappa 0.6) was found for the two graders. Hau *et al* have previously
109 demonstrated that the diagnostic accuracy of IVCN for the diagnosis of microbial
110 keratitis is also affected by the experience of the IVCN grader.¹³ As such there is a
111 need to determine the extent of variability between graders in the clinical setting.
112 Resolution of the IVCN imaging system may also affect the ability of graders to
113 detect pathogens, but to date there have been no formal prospective studies using the
114 higher resolution HRT3 IVCN in the detection of MK.

115

116 In this study, we aim to determine the diagnostic accuracy of HRT3 IVCN in
117 moderate to severe MK in South India using five experienced confocal graders
118 (masked to microbiological diagnosis). We also assess inter and intra-grader
119 agreement.

120

121 **Methods**

122 This study was approved by the Institutional Review Board of Aravind Eye Hospital,
123 Tamil Nadu, India, the Indian Council for Medical Research and the Ethics
124 Committee of the London School of Hygiene and Tropical Medicine. Prior to
125 enrolment in the study, all patients gave written informed consent; study participants
126 who were illiterate gave informed consent with a witnessed thumbprint on the study
127 consent form, as approved by the above Ethics Committees. This study adhered to the
128 tenets of the Declaration of Helsinki and was conducted as per the Standards for
129 Reporting of Diagnostic Accuracy studies (STARD)¹⁴ – see STARD checklist in
130 supplementary Table 1.

131

132 ***Study Participants***

133 This study was based in the Cornea Clinic at Aravind Eye Hospital, Madurai, Tamil
134 Nadu, India. Consecutive patients presenting to the clinic between Feb 2012 and Feb
135 2013 were assessed for eligibility and prospectively enrolled into the study if eligible.
136 The inclusion criteria were: age \geq 18 years, presence of a large corneal ulcer, defined
137 as a stromal infiltrate \geq 3mm in longest diameter, with an overlying epithelial defect
138 and signs of acute inflammation. All eligible patients underwent slit lamp
139 examination by an ophthalmologist (cornea specialist), and relevant clinical
140 history/examination findings were recorded in the standardized study form. We
141 excluded any patients with a descemetocoele or $>$ 80% corneal thinning in the affected
142 eye as assessed on slit lamp examination (i.e. in whom we could not safely applanate
143 the IVCN on to the cornea for imaging), those considered to have herpetic stromal
144 keratitis on clinical grounds (i.e. either a prior history of the disease, or presence of

145 clinical features associated with herpetic disease), or if Snellen visual acuity was
146 worse than 6/60 in the unaffected eye.

147

148 ***IVCM Imaging***

149 The affected eye was anaesthetized using 0.5% proparacaine eyedrops (Aurocaine,
150 Aurolab, Madurai, India) and volume scans of the corneal ulcer were obtained using
151 the HRT3 IVCM (Heidelberg Engineering, Germany) with Rostock Corneal Module,
152 (63x magnification objective lens, Nikon, Japan), by an ophthalmologist trained in
153 performing IVCM and following a standard procedure described elsewhere.¹³ Briefly,
154 volume scans were obtained in the center of the ulcer, and at the 12, 3, 6, and 9
155 o'clock positions of the peripheral ulcer margins. Volume scans were taken from the
156 surface of the ulcer, and manually refocused several times to take progressively
157 deeper overlapping scan sets covering as much of the full depth of the ulcer as
158 possible.

159

160 Immediately after IVCM imaging, the patient underwent scraping of the ulcer base
161 and leading margin for microscopy and culture. The confocal microscopist who
162 performed IVCM imaging was masked to the microbiological diagnosis, but had
163 examined the ulcer at the slit lamp prior to performing IVCM. At the time of image
164 acquisition, this grader (grader 5) was asked to grade the IVCM images for the
165 presence/absence of fungal filaments or acanthamoeba cysts, or if suspicious but not
166 confidently certain then this was graded as the “possible” presence of filaments/cysts.

167

168 ***Microbiological Diagnosis***

169 Immediately after IVCM had been performed and grading recorded, the base and
170 leading edge of the corneal ulcer were scraped using a flame-sterilized Kimura
171 spatula. Scrapings were immediately placed on to two glass slides for light
172 microscopy, and agar plates for culture: blood agar, (BA), potato dextrose agar (PDA)
173 and non-nutrient agar seeded with *E. Coli* in the laboratory if acanthamoeba keratitis
174 was clinically suspected. Standard microbiological methods were followed to detect
175 any pathogen.¹⁵ In brief, slides were stained with 10% potassium hydroxide or gram
176 stain or giemsa to aid visualization of fungal filaments, bacteria or acanthamoeba
177 cysts respectively; agar plates were incubated at 37°C for 2 days for BA, or at 27°C
178 for 7 days for PDA, and were assessed daily for organism growth. A culture was
179 classified as positive if any of the following criteria were satisfied: a) growth of the
180 same species of bacteria or fungus on at least two solid media, or, b) semi-confluent
181 growth at the site of inoculation in one solid medium of an organism that, for bacteria,
182 was the same as the organism identified with gram stain on microscopy. Organism
183 speciation was performed using standard laboratory methods.¹⁵ For fungal
184 identification, spores were stained with lactophenol cotton blue and speciated by the
185 morphological appearance of the colony, hyphae and spores.¹⁶

186

187

188 ***IVCM Grading***

189 Patient-identifying data were removed from all IVCM scans and images were
190 arranged in a random order for each observer to assess. At Moorfields Eye Hospital,
191 our confocal graders assessed all scans of all recruited patients and graded for the
192 definite presence, definite absence or possible presence of fungal filaments or
193 acanthamoeba cysts as described above for grader 5. All graders had varying

194 experience of performing IVCN and grading confocal images for MK, ranging from 6
195 years (graders 1 & 2; grader 2 with an additional 2 years of general IVCN
196 experience), 3.5 years (grader 3), and 2 years (graders 4 & 5 specifically with IVCN
197 MK imaging experience). All graders were masked to the microbiological diagnosis.
198 Graders 1 to 4 were masked to the clinical appearance of the ulcer. Grading data were
199 directly entered into a Microsoft Access database. To measure intra-grader agreement,
200 all image sets were allocated a new random study number and shuffled into a new
201 order. Three graders were able to repeat the grading process at least 3 weeks after the
202 first grading session.

203

204 ***Reference standard***

205 For the purposes of this study the reference for diagnosis of fungus, was a positive
206 culture or (if the culture was negative) the presence of fungal hyphae on light
207 microscopy, as has been used in previous studies.¹⁷ Similarly the reference for
208 acanthamoeba, was a positive culture and/or presence of acanthamoeba cysts on light
209 microscopy; this approach has previously been shown to increase diagnostic accuracy
210 for acanthamoeba detection, compared to use of culture alone.¹⁸ One experienced
211 microbiologist performed the culture and light microscopy interpretation and was
212 masked to the IVCN images and grading, but had a limited clinical history available
213 to them on the microbiology test request form.

214

215 ***Statistical methods***

216 All statistical analyses were performed in Stata 12.1 (StataCorp, Texas, USA).
217 Sample size was estimated as n=200 based on a fungal keratitis prevalence estimate of
218 50%, aiming for sensitivity of 85%, and with marginal error of 7%, as per Hajjan-

219 Tilaki *et al.*¹⁹ Statistical significance of between-group differences in demographic or
220 clinical features was assessed using the Kruskal Wallis test, and chi squared test for
221 proportions. Sensitivity (i.e. ratio of true positives/true positives plus false negatives),
222 specificity (i.e. ratio of true negatives/true negatives plus false positives), positive
223 predictive value (PPV) and negative predictive value (NPV) were calculated using
224 “definite fungus” or “definite acanthamoeba” grades for the primary analysis. The
225 primary outcome measure was the pooled sensitivity and specificity of the 5 graders,
226 calculated along with 95% confidence intervals using a bivariate random-effects
227 regression model that accounts for the correlation between the two measures (*metandi*
228 and *midas* commands in Stata).²⁰⁻²² This is likely to be a conservative estimate since it
229 accounts for the various level of experience of the graders and only 1 grader takes into
230 account the clinical features of the ulcer. Comparison of regraded outcomes with
231 initial grades was performed using the kappa score to calculate intra-grader agreement
232 (to assess reproducibility). A kappa score was also calculated for inter-grader
233 agreement (to assess reliability) for cases graded with certainty as “definite
234 fungus/acanthamoeba” or “no organism seen”. Kappa scores were interpreted as
235 follows: ≤ 0.20 “no agreement”; 0.21-0.39 “minimal agreement”; 0.40-0.59 “weak
236 agreement”; 0.60-0.79 “moderate agreement”; 0.80-0.90 “strong agreement”; >0.90
237 “almost perfect agreement”.²³

238

239 **Results**

240 ***Study Participants:***

241 A total of 254 patients were assessed for study eligibility between February 2012 and
242 February 2013, of whom 13 patients were excluded for history of herpetic keratitis
243 (n=1) or presence of $>80\%$ corneal thinning (n=12). Two patients were also excluded

244 as we were unable to perform diagnostic tests for them: no culture or light microscopy
245 performed (n=1, deep stromal abscess), or total ulcer with no clear cornea to scan
246 with IVCN (n=1) – see supplementary figure for STARD patient flow diagram. A
247 total of 3163 volume scans were obtained with a mean 13 volume scans per patient
248 (range 3-42). A few patients (n=4) were unable to cooperate for the full IVCN
249 imaging protocol and so we were only able to image part of the ulcer - these patients
250 were not excluded. No adverse events were noted from either performing IVCN
251 imaging or corneal scraping for culture/light microscopy.

252

253 Socio-demographic features of the final participants are shown in Table 1. Compared
254 to all others, AK patients had a higher frequency of ring infiltrate (88% in AK vs.
255 31% all others, $p<0.0001$) and a longer median symptom duration (30 days in AK vs.
256 7 days all others, $p<0.0001$).

257

258 ***Microbiological Culture and Light Microscopy Results***

259 Tables 2 and 3 summarize the organisms identified on microbiological testing in the
260 239 patients included in the analysis. The majority of patients (74%, n=176) met the
261 reference standard criteria of fungal positivity. These included 2 cases of mixed
262 infection, i.e. fungal filaments detected on light microscopy but positive culture for
263 bacteria (*Streptococcus viridans* and *Streptococcus pneumoniae* respectively). Thirty
264 participants had fungal filaments detected on light microscopy alone (negative culture
265 for fungus), of whom 83% (n=25) had used antifungal therapy prior to presentation
266 and 50% (n=15) were deep with the stromal infiltrate involving the posterior third of
267 the cornea. All 17 acanthamoeba cases were culture positive and 13 of these were also
268 light microscopy positive (none were solely light microscopy positive for

269 acanthamoeba). The culture positivity rate for any organism was high at 76%
270 (n=182).

271

272 ***Detection of Fungal Filaments by IVCM***

273 Figures 1a and 1b shows an example of fungal filaments as seen in IVCM images of a
274 culture positive fungal ulcer. Overall, all five graders were able to definitely detect
275 fungal filaments in the IVCM images with a pooled sensitivity of 85.7% (95% CI
276 82.2% - 88.6%) and pooled specificity of 81.4% (95% CI 76.0% - 85.9%), with
277 individual grader data shown in Table 4a. Overall, the highest sensitivity (89.8%,
278 95% CI: 84.3%-93.8%) was achieved by the grader with access to the ulcer clinical
279 features (grader 5). The grader with the lowest sensitivity (Grader 2, 79.1%) also had
280 the highest specificity (i.e. fewest false positives). For only the four graders who were
281 masked to clinical features, pooled sensitivity was 84.5% (95% CI: 80.8% – 87.6%)
282 and pooled specificity was 82.0% (95% CI: 75.7% - 86.9%). Earlier presentation with
283 shorter symptom duration (≤ 4 days) had the highest pooled sensitivity for all 5
284 graders of 95% (95% CI: 88 - 98%) but lowest pooled specificity of 53% (95% CI:
285 39% - 66%). As symptom duration increased to longer than 10 days, the pooled
286 sensitivity reduced to 72% (95% CI: 64% - 78%), with concomitant increase in
287 sensitivity to 91% (95% CI: 84% – 95%), as shown in Table 5.

288

289 There was a strong inter-grader agreement between all five masked graders' scores
290 for definite fungus, with a kappa score of 0.88 ($p < 0.0001$). Kappa scores for intra-
291 grader agreement (i.e. test reproducibility) were between 0.88 and 0.95 ($p < 0.0001$),
292 i.e. strong to almost perfect agreement.

293

294 IVCN images for the three culture-positive *Nocardia sp.* cases were classed as not
295 having filamentous structures by 4 out of the 5 graders.

296

297 ***IVCM “false positives” or “false negatives” for fungus***

298 Ten patients were microbiologically negative for fungus but four or more graders
299 categorized these images as showing “definite fungus” (i.e. IVCN “false positives”).

300 Figure 2 shows examples of the fungal branching structures seen in these IVCN
301 images. Nine of these ten ulcers were noted to be deep with extension into the
302 posterior third of the cornea on slit lamp examination and/or IVCN imaging.

303

304 Conversely, nine patients were microbiologically positive for fungus but graded by all
305 5 graders as having no fungal filaments on IVCN (i.e. IVCN “false negatives”). On
306 further IVCN imaging up to 21 days after presentation, fungal filaments were still not
307 detected in five patients and the remaining four patients had progressive corneal
308 thinning or perforation that prevented further IVCN imaging from being performed.

309 Five patients had surface plaques at presentation that caused high reflectivity and
310 difficulty in imaging the ulcer clearly using IVCN. The spectrum of organisms
311 grown from the IVCN false negative ulcers included *Fusarium sp.* (n=4), *Aspergillus*
312 *sp.* (n=3), *Cylindrocarpus sp.* (n=1); in 1 patient no organism was grown but fungal
313 filaments were detected in corneal scrapings on light microscopy for this patient.

314

315 ***IVCM Detection of Acanthamoeba Cysts***

316 For definite detection of acanthamoeba cysts, all five graders had a pooled sensitivity
317 of 88.2% (95% CI 76.2% - 94.6%) and pooled specificity of 98.1% (95% CI 94.9% -
318 99.3%). The four graders masked to clinical features had a very similar pooled

319 sensitivity of 88.5% (95% CI 73.0% - 95.6%) and pooled specificity of 98.0% (95%
320 CI 93.3% - 99.4%). The grader with access to clinical feature data had a sensitivity of
321 88.2%, and specificity of 98.6% (Grader 5, Table 4b). In ulcers presenting earlier (i.e.
322 <20 days symptom duration) the pooled sensitivity and specificity (all 5 graders) was
323 82% (95% CI 34 - 98%) and 98% (95% CI 95 - 99%) respectively. This high
324 sensitivity and specificity was maintained in ulcers with longer symptom duration
325 beyond 30 days (see Table 5).

326

327 For all 5 graders, there was a moderate inter-grader agreement with kappa score 0.72
328 ($p < 0.0001$). Kappa scores for intra-grader agreement for definite *Acanthamoeba* cases
329 ranged from 0.63 to 0.90 ($p < 0.0001$). *Acanthamoeba* cyst morphology is shown in
330 Figure 1c.

331

332 ***IVCM “false positives” or “false negatives” for Acanthamoeba***

333 In the one IVCM “false positive” case, culture and light microscopy were both
334 negative for *acanthamoeba*, but all 5 graders detected *acanthamoeba* cysts on IVCM.
335 Figure 2f shows images from this patient, highlighting the presence of *Acanthamoeba*
336 cyst-like structures.

337

338 There was 1 IVCM “false negative” ulcer, i.e. microbiologically positive for
339 *Acanthamoeba* sp. but no “definite *acanthamoeba*” detected by any grader. Of note,
340 two of the five graders classified the images for this ulcer as “possible
341 *acanthamoeba*”.

342

343 ***“Possible” fungus or acanthamoeba on IVCM***

344 Seventy-one ulcers in total were classified as “possible” fungus present by any grader,
345 with agreement from 3 or more graders on this diagnosis in 7 of these ulcers. The
346 reference standard was fungal positive in 75.3% (n=55) of those graded as “possible
347 fungus”. The remainder either had no growth with no organism on light microscopy
348 (n=9), or were culture/light microscopy positive for *Acanthamoeba sp.* (n=3),
349 *Nocardia sp.* (n=2) or *Streptococcus pneumoniae* (n=2).

350

351 For those classified as “possible acanthamoeba” by any grader (n=75 ulcers), only
352 9.3% were microbiologically positive for acanthamoeba sp. (n=7), the remainder
353 being microbiologically positive for fungus (n=43) or bacteria (n=13), or with no
354 organism detectable on culture or light microscopy (n=12). Three or more graders
355 were in agreement of the “possible acanthamoeba” diagnosis in 13 ulcers of which
356 only 2 were acanthamoeba positive using the reference standard.

357

358 At re-grading, up to 57% of all images initially classified by any grader as possible
359 fungus were shifted to the “definite fungus” category (n=34/60), and 85% of these
360 were reference standard positive for fungus (n=29/34). Of the images initially graded
361 as “possible acanthamoeba”, 9% (n=8/88) were shifted to the “definite
362 acanthamoeba” grade at re-grading, with 75% (n=6/8) of these being
363 microbiologically positive for acanthamoeba. Very few images were converted by any
364 grader from “definite fungus” to “possible fungus” (n=11/438). Six of these images
365 were converted by at least 2 of the 3 graders (*Curvularia sp.* n=2, *Fusarium sp.* n=2,
366 culture/light microscopy negative, n=2) and the remaining images were culture
367 positive for *Aspergillus flavus* (n=2), *Fusarium sp.* (n=1), *Nocardia sp.* (n=1) or
368 culture/light microscopy negative (n=1). For acanthamoeba, again few images were

369 regraded from “definite” to “possible” (n=9/58), with 8 images converted by at least 2
370 of 3 graders (4 culture positive for *Acanthamoeba sp.*, 2 for *Fusarium sp.*, 2 for
371 *Nocardia sp.*), and the remaining one culture positive for *Fusarium sp.*

372

373 **Discussion**

374 Large corneal ulcers can present a major diagnostic challenge, especially as they often
375 have mixed or atypical clinical features and may be culture negative. Delays in
376 treatment of fungal and acanthamoeba keratitis in particular can lead to significant
377 visual loss, and even loss of the eye.²⁴⁻²⁶ IVCM is a non-invasive method through
378 which fungal filaments and acanthamoeba cysts can be immediately detected in the
379 patient’s cornea,⁹ allowing the clinician to promptly start the correct antimicrobial
380 therapy. In 2004, the American Academy of Ophthalmology conducted an evidence-
381 based assessment of the value of IVCM as a diagnostic tool for MK. With only level
382 II and III evidence available at that time, they concluded that IVCM could be useful
383 as an adjunctive test in fungal keratitis, but for acanthamoeba keratitis there was
384 sufficient evidence to support the use of IVCM as the sole diagnostic test.²⁷ Since
385 then, two prospective studies using the Confoscan IVCM have found a high
386 sensitivity and specificity for the detection of fungal filaments and acanthamoeba
387 cysts.^{11,12} In this report, we provide for the first time evidence of a high diagnostic
388 accuracy of the HRT3 confocal microscope in the detection of fungi and
389 acanthamoeba in moderate to severe MK in a clinical setting, comparable to the
390 results found in these previous two studies. Use of a multi-grader approach allowed
391 for a more accurate assessment of sensitivity and specificity. Our study demonstrated
392 a slightly higher sensitivity for detection of acanthamoeba than fungal filaments
393 compared to the study by Vaddavalli *et al.* We were only able to study a small

394 number of participants with acanthamoeba keratitis, and so further research is
395 required with larger study population, as well as earlier stages of disease, to more
396 fully evaluate the HRT3 IVCN for the diagnosis of acanthamoeba keratitis.

397

398 We have found that experienced IVCN graders were able to detect fungi or
399 acanthamoeba in 94.8% of all culture and/or light microscopy positive ulcers. The
400 main cause of IVCN “false negatives” was technical difficulty in being able to obtain
401 adequate IVCN images. Ulcers with superficial plaques caused a high level of surface
402 reflectivity in the IVCN images, thus inhibiting recognition of fungal filaments in the
403 ulcer surface or margins, as we found in five of our nine IVCN “false negative”
404 fungal ulcers. A small number of patients were only able to tolerate IVCN imaging
405 for a short time period and so only a limited number of images were obtained and
406 these images may not have captured pathogens present in deeper aspects of the ulcer.
407 False negatives due to poor patient cooperation have been previously reported with
408 this imaging modality.²⁸ In the case of our 11 IVCN “false negatives”, the clinical
409 features as well as microbiological results in these patients were able to guide
410 appropriate treatment. Other reasons for IVCN “false negatives” include the learning
411 curve for the IVCN operator in adequately scanning the whole ulcer to capture any
412 pathogen in the images, as well as the presence of a high degree of stromal
413 inflammation that could mask the presence of the pathogen (i.e. through high
414 reflectivity reducing image contrast as with surface plaques, or difficulty in
415 identifying acanthamoeba cysts in the presence of a large number of white cells since
416 they both have similar morphology).

417

418 We have found that IVCN graders were able to detect a pathogen in 11 culture and
419 light microscopy negative ulcers. The IVCN images in these ulcers had classical
420 features of fungal hyphae or acanthamoeba cysts and so we feel these represent true
421 cases of disease. In the majority of patients, these ulcers were deep, involving the
422 posterior third of the cornea and therefore making it less likely that superficial corneal
423 scraping would collect viable fungi to grow in culture or to be seen on light
424 microscopy. In such cases, IVCN is an invaluable tool to rapidly detect fungal
425 filaments in the deep stroma and allows the correct antimicrobial treatment to be
426 commenced without the need for invasive corneal biopsy to identify the pathogen.²⁹
427 Other causes of a “false positive” IVCN for fungus include the presence of other
428 linear branching structures such as corneal nerves, and *Nocardia sp.* filaments.³⁰ Only
429 1 grader out of 5 classified images from *Nocardia keratitis* as containing fungal
430 filaments in this study. Since *Nocardia sp.* filaments are thinner in diameter than
431 filamentous fungi (<1.5 microns versus 3-6 microns resp.),³¹ they can be more
432 difficult to detect on IVCN particularly in the presence of significant stromal oedema
433 or inflammation as in moderate to severe keratitis, but were readily detected
434 microbiologically in our study.

435

436 In the clinical setting, an uncertain IVCN test result can cause concern with regards to
437 which antimicrobial therapy to commence. On further analysis of all images graded as
438 showing “possible” presence of a pathogen, 75% of those graded as “possible fungus”
439 were appropriately classified when compared with the reference standard, but less
440 than 10% of the images graded as “possible acanthamoeba” corresponded to
441 microbiologically confirmed acanthamoebal ulcers. This finding confirms the
442 importance of adding clinical examination and microbiological testing to IVCN

443 imaging to reach a definite diagnosis for acanthamoebal infection in particular, rather
444 than using one diagnostic tool alone, as also found by others.¹⁸

445

446 There was an apparent improvement in the certainty of diagnosis on re-grading
447 images. This learning effect was also detected by Hau *et al*, who found that the
448 specificity improved for all graders upon IVCN MK image re-grading at a later
449 date.¹³ They also found that as the level of IVCN experience of the grader
450 increased,¹³ the diagnostic accuracy for detection of MK also improved, thus
451 indicating the importance of training in IVCN image recognition for all new graders.
452 The IVCN grader may also benefit from having access to a clinical image of the
453 ulcer,¹⁸ since our grader with access to clinical feature information had a higher
454 sensitivity for fungal detection.

455

456 In this study, although the graders were from a variety of backgrounds (ophthalmic
457 nurses, optometrists and ophthalmologists) and levels of experience, they had a high
458 inter-grader agreement for pathogen detection. We found higher kappa scores for
459 inter-grader agreement than Vaddavalli *et al*,¹² which may be due to the higher
460 resolution of the HRT3 imaging system allowing for higher definition images of the
461 pathogen, as well as the training/experience of our confocal graders with this high
462 resolution imaging system. Intra-observer agreement was in our study was also high,
463 and was better for fungal detection with the best agreement in the most experienced
464 observer.

465

466 Limitations of this study include the dominance of filamentary fungal keratitis, and
467 the relatively low proportion of bacterial infections. We were unable to study confocal

468 appearances of candida keratitis, which is more common in more temperate climates.
469 We only studied 17 cases of acanthamoeba, and so further research is needed to more
470 fully elucidate acanthamoebal detectability on IVCN imaging in a larger study. The
471 cost of the confocal microscope may be too high for its routine uptake in areas with
472 the highest endemicity for fungal and acanthamoeba keratitis, in low and middle
473 income countries in tropical regions; however, delay in treatment may result in a
474 greater cost in the long term due to poorer visual outcome related to delayed
475 diagnosis.

476

477 There was a high culture positive rate in this study. We believe there are a number of
478 reasons for this, in addition to our inclusion of mainly larger ulcers. Firstly, we used a
479 microbiology service that is particularly optimized for ocular microbiology. Secondly,
480 culture could be initiated with very little delay after sample collection since the
481 laboratory is situated next to the Cornea Clinic at Aravind Eye Hospital. Thirdly, the
482 standard practice is to use a kimura spatula, which we also believe gives a more
483 ample sample than using a needle, thereby improving the organism detection rate. In
484 regions with lower culture positivity rates, the value of IVCN may be greater, as a
485 higher proportion of cases will be culture negative. Although our study has focused
486 on larger ulcers, we still found that IVCN can detect fungi with a high sensitivity in
487 ulcers with only a few days' symptom duration. Also, for acanthamoeba detection
488 with IVCN, we found a high sensitivity and specificity for both early and late
489 presenting ulcers.

490

491 In summary, we have found that experienced graders are able to detect fungal or
492 acanthamoebal elements within HRT3 IVCN images with high sensitivity, specificity

493 and test reproducibility in moderate to severe keratitis. This imaging modality
494 outperforms standard microbiological methods for deep ulcers in particular. The
495 addition of clinical feature data improved diagnostic accuracy. IVCN may therefore
496 be considered as an adjunctive tool, in addition to clinical examination and
497 microbiological testing, for detection of fungi or acanthamoeba in microbial keratitis.

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499

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501 **References**

- 502 1. Whitcher, J.P. & Srinivasan, M. Corneal ulceration in the developing world-
503 -a silent epidemic. *The British journal of ophthalmology* 1997;81:622-
504 623.
- 505 2. Tu, E.Y. & Joslin, C.E. Recent outbreaks of atypical contact lens-related
506 keratitis: what have we learned? *Am J Ophthalmol* 2010;150:602-608
507 e602.
- 508 3. Dalmon, C., Porco, T.C., Lietman, T.M., et al. The clinical differentiation of
509 bacterial and fungal keratitis: a photographic survey. *Invest Ophthalmol*
510 *Vis Sci* 2012;53:1787-1791.
- 511 4. Lalitha, P., Prajna, N.V., Manoharan, G., et al. Trends in bacterial and fungal
512 keratitis in South India, 2002-2012. *The British journal of ophthalmology*
513 2014.
- 514 5. Burton, M.J., Pithuwa, J., Okello, E., et al. Microbial keratitis in East Africa:
515 why are the outcomes so poor? *Ophthalmic epidemiology* 2011;18:158-
516 163.
- 517 6. Asbell, P. & Stenson, S. Ulcerative keratitis. Survey of 30 years' laboratory
518 experience. *Arch Ophthalmol* 1982;100:77-80.
- 519 7. Wahl, J.C., Katz, H.R. & Abrams, D.A. Infectious keratitis in Baltimore.
520 *Annals of ophthalmology* 1991;23:234-237.
- 521 8. Gopinathan, U., Sharma, S., Garg, P., et al. Review of epidemiological
522 features, microbiological diagnosis and treatment outcome of microbial
523 keratitis: experience of over a decade. *Indian journal of ophthalmology*
524 2009;57:273-279.

- 525 9. Labbe, A., Khammari, C., Dupas, B., et al. Contribution of in vivo confocal
526 microscopy to the diagnosis and management of infectious keratitis. *Ocul*
527 *Surf* 2009;7:41-52.
- 528 10. Zhivov, A., Stachs, O., Stave, J., et al. In vivo three-dimensional confocal
529 laser scanning microscopy of corneal surface and epithelium. *Br J*
530 *Ophthalmol* 2009;93:667-672.
- 531 11. Kanavi, M.R., Javadi, M., Yazdani, S., et al. Sensitivity and specificity of
532 confocal scan in the diagnosis of infectious keratitis. *Cornea* 2007;26:782-
533 786.
- 534 12. Vaddavalli, P.K., Garg, P., Sharma, S., et al. Role of confocal microscopy in
535 the diagnosis of fungal and acanthamoeba keratitis. *Ophthalmology*
536 2011;118:29-35.
- 537 13. Hau, S.C., Dart, J.K.G., Vesaluoma, M., et al. Diagnostic accuracy of
538 microbial keratitis with in vivo scanning laser confocal microscopy.
539 *British Journal of Ophthalmology* 2010;94:982-987.
- 540 14. Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., et al. STARD 2015: an updated list
541 of essential items for reporting diagnostic accuracy studies. *BMJ*
542 2015;351:h5527.
- 543 15. Wilhelmus, K.R., Liesegang, T.J., Osato, M.S., et al. *Cumitech 13A: laboratory*
544 *diagnosis of ocular infections*, (American Society of Microbiology Press,
545 Washington DC, 1994).
- 546 16. Thomas, P.A. Current Perspectives on Ophthalmic Mycoses. *Clinical*
547 *Microbiology Reviews* 2003;16:730-797.

- 548 17. Prajna, N.V., Krishnan, T., Mascarenhas, J., et al. The mycotic ulcer
549 treatment trial: a randomized trial comparing natamycin vs voriconazole.
550 JAMA ophthalmology 2013;131:422-429.
- 551 18. Tu, E.Y., Joslin, C.E., Sugar, J., et al. The relative value of confocal
552 microscopy and superficial corneal scrapings in the diagnosis of
553 Acanthamoeba keratitis. Cornea 2008;27:764-772.
- 554 19. Hajian-Tilaki, K. Sample size estimation in diagnostic test studies of
555 biomedical informatics. J Biomed Inform 2014;48:193-204.
- 556 20. Reitsma, J.B., Glas, A.S., Rutjes, A.W., et al. Bivariate analysis of sensitivity
557 and specificity produces informative summary measures in diagnostic
558 reviews. Journal of clinical epidemiology 2005;58:982-990.
- 559 21. Harbord, R.M. & Whiting, P. metandi: Meta-analysis of diagnostic accuracy
560 using hierarchical logistic regression. The Stata Journal 2009;9:211-229.
- 561 22. Dwamena, B.A. MIDAS: Stata Module for Meta-analytical Integration of
562 Diagnostic Test Accuracy Studies. (Boston College Department of
563 Economics, Chestnut Hill, MA, USA, 2009).
- 564 23. McHugh, M.L. Interrater reliability: the kappa statistic. Biochem Med
565 (Zagreb) 2012;22:276-282.
- 566 24. Burton, M.J., Pithuwa, J., Okello, E., et al. Microbial keratitis in East Africa:
567 why are the outcomes so poor? Ophthalmic Epidemiol 2011;18:158-163.
- 568 25. Lalitha, P., Prajna, N.V., Kabra, A., et al. Risk factors for treatment outcome
569 in fungal keratitis. Ophthalmology 2006;113:526-530.
- 570 26. Bacon AS, D.J., Ficker LA, Matheson MM, Wright P. Acanthamoeba
571 keratitis. The value of early diagnosis. Ophthalmology 1993;100:1238-
572 1243.

- 573 27. Kaufman, S.C., Musch, D.C., Belin, M.W., et al. Confocal microscopy: a
574 report by the American Academy of Ophthalmology. *Ophthalmology*
575 2004;111:396-406.
- 576 28. Parmar, D.N., Awwad, S.T., Petroll, W.M., et al. Tandem scanning confocal
577 corneal microscopy in the diagnosis of suspected acanthamoeba keratitis.
578 *Ophthalmology* 2006;113:538-547.
- 579 29. Das, S., Samant, M., Garg, P., et al. Role of confocal microscopy in deep
580 fungal keratitis. *Cornea* 2009;28:11-13.
- 581 30. Chiou, A.G., Kaufman, S.C., Beuerman, R.W., et al. Differential diagnosis of
582 linear corneal images on confocal microscopy. *Cornea* 1999;18:63-66.
- 583 31. Vaddavalli, P.K., Garg, P., Sharma, S., et al. Confocal microscopy for
584 *Nocardia* keratitis. *Ophthalmology* 2006;113:1645-1650.
- 585
- 586

587 **Figure Legends**

588

589 Figure 1: *In vivo* confocal microscopy images (IVCM) of *Fusarium sp.* culture-
590 positive ulcer showing overlapping fungal filaments in the centre of the ulcer (A), and
591 more distinct fungal filaments at the periphery (B); IVCM images of an
592 *Acanthamoeba sp.* culture-positive ulcer showing cysts in chains and clusters (C).

593

594 Figure 2: *In vivo* confocal microscopy images of six culture and light microscopy
595 negative ulcers in which graders detected fungal filaments (A-E) or acanthamoeba
596 cysts (F). Note the similarity of cyst appearance to those in Figure 1 image C with a
597 similar absence of inflammatory cell infiltrate in the corneal stroma.

598

599 Supplementary Figure: Flow of participants through the study (STARD diagram)

600

601 **Table Legends**

602

603 Table 1: Demographic data and clinical features of study participants

604 Table 2: Distribution of organisms identified by culture and/or light microscopy

605 Table 3: Species cultured for fungi (n=144) and bacteria (n=21)

606 Table 4a: Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative
607 Predictive value (NPV) for definite detection of fungi on

608 Table 4b: Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative
609 Predictive value (NPV) for definite detection of

610

611 Supplementary Table: STARD Checklist

Table 1: Demographic data and clinical features of study participants

	Fungal Keratitis (74%, n=176) ^a	Acanthamoeba Keratitis (7%, n=17)	Bacterial Keratitis (8%, n=19)	Culture/light microscopy negative (11%, n=27)	p-value
Median Age, years (range)	50 (19 - 80)	40 (23 - 70)	57 (19 - 80)	50 (22 - 74)	0.3166
Male Gender, n (%)	116 (65.9%)	10 (58.8%)	11 (57.9%)	16 (59.3%)	0.7909
Symptom duration: median no. of days (range)	7 (1 - 90)	30 (4 - 155)	7.5 (2 - 20)	8 (2 - 60)	0.0001
Prior antibiotic use, n (%) ^b	112 (72.3%)	14 (87.5%)	13 (81.3%)	14 (63.6%)	0.3509
Prior antifungal use, n (%) ^b	89 (57.4%)	10 (62.5%)	7 (43.8%)	13 (59.1%)	0.7965
Ring infiltrate	52 (29.6%)	15 (88.2%)	10 (52.6%)	7 (25.9%)	0.0001

^a Mixed infections included (culture positive for bacteria but microscopy positive for fungus, n=2

^b For prior drug use, n=209 (data not available for 30 patients)

Table 2: Distribution of organisms identified by culture and/or light microscopy

<i>Culture positives (n=182)</i>	N	%
Acanthamoeba	17	7.1%
Fungi	144	60.3%
Bacteria	19	9.6%
Mixed: Culture +ve for bacteria, microscopy +ve for fungi	2	0.8%
<i>Culture negatives (n=57)</i>		
Culture negative but light microscopy positive for fungus	30	12.6%
Culture negative but light microscopy positive for bacteria	4	1.7%
Culture negative and light microscopy negative	23	9.6%
<i>Total</i>	239	100%

Table 3: Species cultured for fungi (n=144) and bacteria (n=21)

Organism	Species	N	%
Fungi: Hyaline	<i>Fusarium sp.</i>	73	50.7%
	<i>Aspergillus flavus</i>	26	18.1%
	<i>Aspergillus fumigatus</i>	5	3.5%
	<i>Aspergillus terreus</i>	2	1.4%
	<i>Cylindrocarpon sp.</i>	1	0.7%
	<i>Unidentified hyaline fungi</i>	14	9.7%
Fungi: Dematiaceous	<i>Curvularia sp.</i>	5	3.5%
	<i>Exserohilum sp.</i>	4	2.8%
	<i>Lasiodiplodia sp.</i>	2	1.4%
	<i>Bipolaris sp.</i>	1	0.7%
	<i>Unidentified dematiaceous fungi</i>	11	7.6%
Bacteria: Gram positives	<i>Streptococcus pneumoniae</i>	10	47.6%
	<i>Streptococcus viridans</i>	3	14.3%
	<i>Staphylococcus epidermidis</i>	2	9.5%
	<i>Nocardia sp.</i>	3	14.3%
Bacteria: Gram negatives	<i>Pseudomonas aeruginosa</i>	2	9.5%
	<i>Aeromonas sp.</i>	1	4.8%

Table 4a: Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive value (NPV) for definite detection of fungi on IVCM compared to Culture and/or Light Microscopy

Grader	N*	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
1	219	139	49	9	22	86.3 (80 - 91.2)	84.5 (72.6 - 92.7)	93.9 (88.8 - 97.2)	69.0 (56.9 - 79.5)
2	217	121	55	9	32	79.1 (71.8 - 85.2)	85.9 (75.0 - 93.4)	93.1 (87.3 - 96.8)	63.2 (52.2 - 73.3)
3	190	117	44	9	20	85.4 (78.4 - 90.8)	83.0 (70.2 - 91.9)	92.9 (86.9 - 96.7)	68.8 (55.9 - 79.8)
4	224	145	42	15	22	86.8 (80.7 - 91.6)	73.7 (60.3 - 84.5)	90.6 (85.0 - 94.7)	65.6 (52.7 - 77.1)
5^a	239	158	50	13	18	89.8 (84.3 - 93.8)	79.4 (67.3 - 88.5)	92.4 (87.4 - 95.9)	73.5 (61.4 - 83.5)

Abbreviations: TP=True Positive, TN=True Negative, FP=False Positive, FN=False Negative

* The total no. of patients classified as having "Possible fungus" by each grader and therefore excluded from this analysis are as follows:

Grader 1 (n=21), Grader 2 (n=23), Grader 3 (n=49), Grader 4 (n=16), Grader 5 (n=1)

^a Grader 5 was unmasked to ulcer clinical features.

Table 4b: Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive value (NPV) for definite detection of acanthamoeba on IVCIM compared to Culture and/or Light Microscopy

Grader	N*	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
1	208	11	187	9	1	91.7 (61.5 - 99.8)	95.4 (91.5 - 97.9)	55.0 (31.5 - 76.9)	99.5 (97.1 - 100)
2	202	12	188	1	1	92.3 (64.0 - 99.8)	99.5 (97.1 - 100)	92.3 (64.0 - 99.8)	99.5 (97.1 - 100)
3	205	12	191	1	1	92.3 (64.0 - 99.8)	99.5 (97.1 - 100)	92.3 (64.0 - 99.8)	99.5 (97.1 - 100)
4	218	12	188	14	4	75.0 (47.6 - 92.7)	93.1 (88.6 - 96.2)	46.2 (26.6 - 66.6)	97.9 (94.8 - 99.4)
5^a	239	15	219	3	2	88.2 (63.6 - 98.5)	98.6 (96.1 - 99.7)	83.3 (58.6 - 96.4)	99.1 (96.8 - 99.9)

Abbreviations: TP=True Positive, TN=True Negative, FP=False Positive, FN=False Negative

*The total no. of patients classified as having "Possible acanthamoeba" by each grader and therefore excluded from this analysis are as follows: Grader 1 (n=31), Grader 2 (n=37), Grader 3 (n=32), Grader 4 (n=21); 2 patients excluded by Grader 3 as having "ungradeable images".

^a Grader 5 was unmasked to ulcer clinical features.

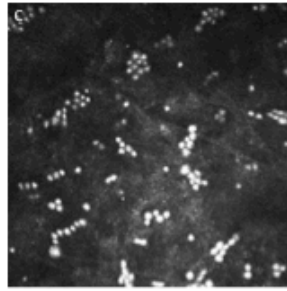
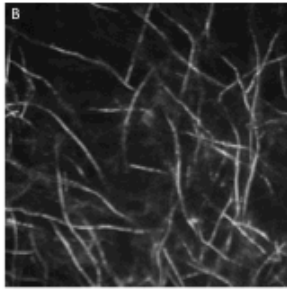
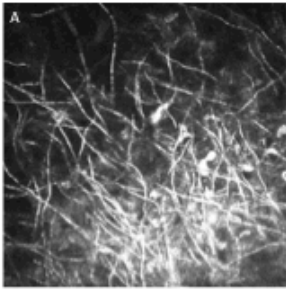
Table 5: Pooled Sensitivity and Specificity for all 5 graders by Symptom Duration (Split by Quartile for Fungi, by Median for Acanthamoeba)

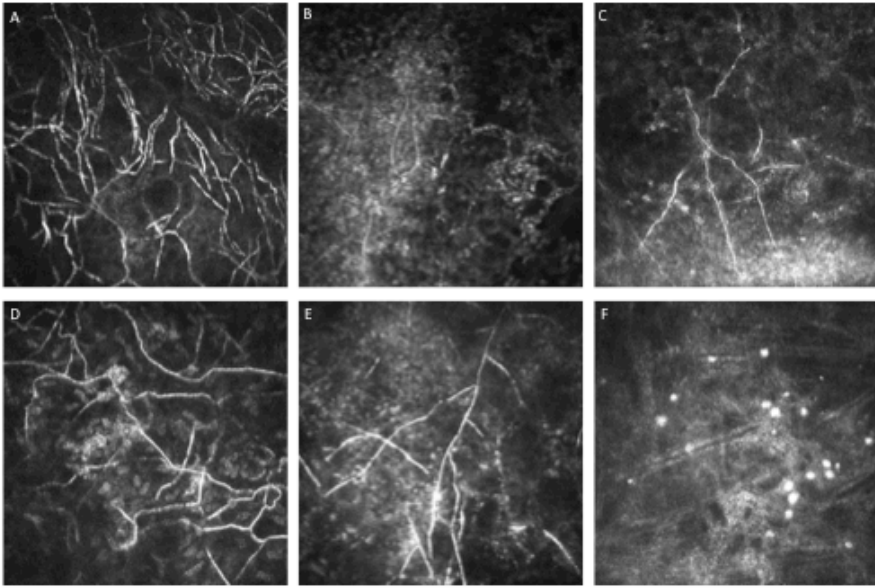
Organism	Symptom Duration	Sensitivity (%)	Specificity (%)
Fungal Keratitis	Q1: ≤4 days	95 (88-98)	53 (39-66)
	Q2: 5-7 days	86 (81-90)	75 (64-84)
	Q3: 8-10 days	91 (85-95)	96 (84-99)
	Q4: >10 days	72 (64-78)	91 (84-95)
Acanthamoeba Keratitis	Q1: <20 days	82 (34-98)	98 (95-99)
	Q2: 20-30 days	98 (53-100)	96 (76-100)
	Q3&4: >30 days	83 (68-92)	96 (76-99)

Supplemental Table - Online Only

SUPPLEMENTARY TABLE: STARD CHECKLIST

Section & Topic	No	Item	Reported on page #
TITLE OR ABSTRACT			
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	1
ABSTRACT			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	2-3
INTRODUCTION			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	4-5
	4	Study objectives and hypotheses	5
METHODS			
<i>Study design</i>	5	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	6
<i>Participants</i>	6	Eligibility criteria	6
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	6
	8	Where and when potentially eligible participants were identified (setting, location and dates)	6
	9	Whether participants formed a consecutive, random or convenience series	6
<i>Test methods</i>	10a	Index test, in sufficient detail to allow replication	7-10
	10b	Reference standard, in sufficient detail to allow replication	7-10
	11	Rationale for choosing the reference standard (if alternatives exist)	9-10
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	9-10
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	9
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test	8-9
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	8-9
<i>Analysis</i>	14	Methods for estimating or comparing measures of diagnostic accuracy	9-10
	15	How indeterminate index test or reference standard results were handled	10
	16	How missing data on the index test and reference standard were handled	9-10
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	9-10
	18	Intended sample size and how it was determined	9
RESULTS			
<i>Participants</i>	19	Flow of participants, using a diagram	STARD Flow Chart
	20	Baseline demographic and clinical characteristics of participants	10-11, Table 1
	21a	Distribution of severity of disease in those with the target condition	10-11, Table 1
	21b	Distribution of alternative diagnoses in those without the target condition	STARD Flow Chart, Tables 1-3
	22	Time interval and any clinical interventions between index test and reference standard	7
<i>Test results</i>	23	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	Tables 4a and 4b
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	11-13
	25	Any adverse events from performing the index test or the reference standard	10
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	17-18
	27	Implications for practice, including the intended use and clinical role of the index test	18
OTHER INFORMATION			
	28	Registration number and name of registry	n/a
	29	Where the full study protocol can be accessed	PhD Thesis LSHTM
	30	Sources of funding and other support; role of funders	1





Supplementary Figure: Flow of Participants through the Study (STARD diagram)

