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# **Diagnostic accuracy of microbial keratitis with *in vivo* scanning laser confocal microscopy**

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## **ABSTRACT**

### **Aims**

To determine the accuracy of diagnosing microbial keratitis by masked medical and non-medical observers using the Heidelberg Retina Tomograph II / Rostock Cornea Module *in vivo* confocal microscope.

### **Methods**

Confocal images were selected for 62 eyes with culture or biopsy proven infections. The cases comprised of 26 *Acanthamoeba*, 12 fungus, 3 *Microsporidia*, 2 *Nocardia*, and 19 bacterial infections (controls). The reference standard for comparison was a positive tissue diagnosis. These images were assessed on two separate occasions by 4 observers who were masked to the tissue diagnosis. Diagnostic accuracy indices, Kappa ( $\kappa$ ) statistic and percentage agreement values were calculated. The Spearman correlation coefficient ( $r_s$ ) was calculated for the number of correct diagnoses versus duration of disease.

### **Results**

The highest sensitivity and specificity values were 55.8% and 84.2%, and the lowest sensitivity and specificity values were 27.9% and 42.1%. The highest positive and negative likelihood ratios were 2.94 and 0.59, respectively. Agreement values were: fair to moderate ( $\kappa$ , 0.22-0.44) for reference standard versus observer diagnosis, moderate to good in intra-observer variability (repeatability,  $\kappa$  0.56-0.88), and poor to moderate in inter-observer variability (reproducibility,  $\kappa$ , 0.15-0.47). The correct

diagnosis was associated with duration of disease for *Acanthamoeba* keratitis ( $r_s = 0.60$ ,  $p = 0.001$ ).

### **Conclusions**

The diagnostic accuracy of microbial keratitis by confocal microscopy is dependent on observer experience. Intra-observer repeatability was better than inter-observer reproducibility. Difficulty in distinguishing host cells from pathogenic organisms limits the value of confocal microscopy as a stand-alone tool in diagnosing microbial keratitis.

## 1 INTRODUCTION

2

3 Difficulties in clinical and microbiological diagnosis are one of the major problems in  
4 the management of microbial keratitis particularly when caused by protozoa  
5 (*Acanthamoeba* and *Microsporidia*), fungi or filamentary bacteria. Diagnosis of these  
6 pathogens is difficult as they often take days or weeks to grow in culture and, in any  
7 case, culture is insensitive with culture positive rates rarely exceeding 60%.[1]  
8 Although culture is still the primary diagnostic tool in tertiary referral centres it is not  
9 widely available to many patients because of limited resources.

10

11 The confocal microscope allows detailed *in vivo* analysis of normal[2] and  
12 pathological corneas. In patients with presumed corneal infection, it is used in  
13 diagnosis and in examination of the extent of involvement of tissue by infection and  
14 associated inflammation. All published studies have been directed at diagnosis and a  
15 number have shown both white light and laser confocal microscopy to be effective in  
16 diagnosing *Acanthamoeba*,[3-5] fungal,[6-8] *Nocardia*[9] and *Microsporidia*  
17 keratitis.[10] However, these studies only present case series or reports and there are  
18 limited published data on evaluating the diagnostic accuracy of confocal microscopy.  
19 Two recent studies have found high sensitivity and specificity values for diagnosing  
20 fungal keratitis (FK) and *Acanthamoeba* keratitis (AK) with the Confoscan 3.0 (Nidek  
21 Technology, Padova, Italy).[11,12] However, factors such as observer or selection  
22 bias, the absence of masking the observers from the microbiological diagnosis, and  
23 lack of appropriate controls may have resulted in overestimates of the sensitivity and  
24 specificity values. Although experience in interpreting confocal keratitis images is  
25 essential, the accuracy of diagnosing microbial keratitis by clinicians with differing

26 levels of confocal microscopy experience and the potential of using trained  
27 technicians in interpreting images have not previously been assessed. These are  
28 important considerations in evaluating this technique. The aim of this study was to  
29 examine the diagnostic accuracy of microbial keratitis with the Heidelberg Retina  
30 Tomograph II / Rostock Cornea Module (HRT II/RCM) *in vivo* confocal microscope,  
31 as a stand-alone tool, by trained medical and non-medical observers with differing  
32 confocal microscopy experience.

33

## 34 **MATERIALS AND METHODS**

35

### 36 **Patients**

37 This study was approved by the Research & Ethics Committee of Moorfields Eye  
38 Hospital and it adhered to the tenets of Declaration of Helsinki. We retrospectively  
39 reviewed the case notes of a consecutive series of microbial keratitis patients who had  
40 had both corneal cultures or corneal biopsy and confocal microscopy (n=105) from  
41 January 1, 2005 to January 4, 2008. These cases were both those refractory to  
42 conventional treatment and those with unusual clinical features such as perineural  
43 infiltrates and ring infiltrates. Patients were either referred from Moorfields  
44 Emergency Department or from other institutions. Of the 105 cases, 62 culture or  
45 biopsy positive cases (62 eyes) were identified: 26 *Acanthamoeba*, 11 fungus, 1  
46 fungus and bacteria, 3 *Microsporidia*, 2 *Nocardia*, and 19 bacteria. Bacteria were used  
47 as controls because they are normally too small to detect with confocal microscopy,  
48 [9,13] therefore the case which was culture positive for both fungus and bacteria was  
49 classified as a fungal keratitis for the purposes of the study. We did not classify

50 *Nocardia* as controls because they are filamentous bacteria and can form filamentous  
51 structures that are large enough to be distinguished by confocal microscopy.[9]  
52 Empirical treatments started prior to assessment in this study included topical  
53 antimicrobial agents and topical steroids for presumed herpes, bacterial or keratitis of  
54 unknown cause, respectively. Irrespective of the referring diagnosis, all patients had  
55 undergone a full clinical examination by a corneal specialist and repeat corneal  
56 scraping for culture and confocal microscopy on the same day. If the scraping was  
57 culture negative, and the keratitis progressive, then a corneal biopsy was later  
58 performed. Exclusion criteria were culture or biopsy negative keratitis cases, and  
59 patients who declined to have confocal microscopy or a corneal culture as part of their  
60 clinical investigation. The reference standard for this study was a diagnosis either by  
61 isolation on culture of a corneal scraping or histological diagnosis on a corneal biopsy;  
62 other ancillary culture sources such as contact lens case and solutions were not used.  
63 The clinical outcomes were recorded for all the patients in the study and were  
64 consistent with the diagnosis based on culture or histology therefore it is unlikely, but  
65 possible, that there was unrecognised polymicrobial infections which may have been  
66 identified on confocal but not by culture or biopsy. We followed the Standards for  
67 Reporting of Diagnostic accuracy (STARD) initiative in conducting this study.[14]

68

### 69 **Culture and biopsy methods**

70 Corneal scrapings for microbial culture were inoculated on the following media:  
71 blood agar, Sabouraud's dextrose agar (fungi), Robertson's cooked meat (anaerobic  
72 bacteria), *Escherichia coli*-seeded non-nutrient agar (*Acanthamoeba*), brain heart  
73 infusion (fastidious organisms, fungi) and Lowenstein-Jensen (mycobacteria,  
74 *Nocardia*). Scrapings were smeared on sterile glass slides for Gram and Giemsa stains.



75 All microbiological investigations were undertaken independently in an external  
76 laboratory. For biopsy a superficial lamellar disc of the affected cornea was trephined  
77 under local anaesthetic to provide a further specimen for microbiology and  
78 histopathological staining.

79

#### 80 **Confocal microscopy measurement protocol**

81 *In vivo* confocal microscopy was performed on all 62 eyes by a single experienced  
82 observer (SH) with the HRT II / RCM (Heidelberg Engineering GmbH, Dossenheim,  
83 Germany) confocal microscope following a Standard Operating Procedure as follows.  
84 A sterile Tomocap (Heidelberg Engineering GmbH, Dossenheim, Germany) was  
85 mounted over the objective of the microscope (Zeiss, x 63), and Polyacrylic acid 0.2%  
86 (Viscotears, Novartis) was used as a coupling agent between the cap and the lens  
87 objective. Topical anaesthetic (Proxymetacaine hydrochloride 0.5%, Chauvin) and  
88 Carmellose sodium 1% (Celluvisc, Allergan) was instilled into both eyes to provide  
89 comfort and act as a coupling fluid between the front of the Tomocap and the cornea.  
90 Options for image acquisition include section (a single image at a particular depth),  
91 volume (a series of images over 60µm depth) and sequence scans (a video sequence at  
92 a particular depth). The volume scan option was selected for image acquisition  
93 because it allowed the capturing of large number of images over a short space of time.  
94 The central region of the corneal ulcer or corneal infiltrate was scanned first followed  
95 by the top, left, bottom and right margin of the lesion. At each point, the epithelial  
96 layer of the affected area was scanned first and the focal plane of the microscope  
97 adjusted until the whole depth of the ulcer or infiltrate had been scanned. When there  
98 was more than one infiltrate, the same scanning sequence was repeated for each  
99 infiltrate. The wavelength of the laser employed in the HRT II / RCM is 670 nm and

100 each standard 2 dimensional image consists of 384 x 384 pixels covering an area of  
101 400  $\mu\text{m}$  x 400  $\mu\text{m}$ . The axial resolution is 7.6 $\mu\text{m}$ ; compared to other instruments such  
102 as the Tandem scanning microscope (9 $\mu\text{m}$ ) and ConfoScan 4 (29 $\mu\text{m}$ ).[15]

103

#### 104 **Image selection**

105 The confocal images of all the scans were reviewed by two experienced confocal  
106 microscopist (SH and JD). In diagnosing keratitis, a considerable amount of time is  
107 often needed to find an image that would yield sufficient information to be able to  
108 identify the organism. This is due to masking of the organisms by the cellular  
109 inflammatory response and that they seldom distribute evenly within the cornea  
110 during active infection. Therefore, to ensure all our observers had the maximum  
111 likelihood in diagnosing the type of keratitis, the best quality 384 x 384 pixel  
112 resolution digital image indicating clearly the culture proven pathogen from the  
113 corneal ulcer or infiltrate was selected and exported onto Microsoft Power Point<sup>®</sup>  
114 (Microsoft Corp., Redmond, WA, USA). These included those of *Acanthamoeba* -  
115 round single or double walled hyper-reflective objects (~10-20  $\mu\text{m}$ ) consistent with  
116 *Acanthamoeba* cysts,[4,5] fungus - linear irregular branching hyper-reflective objects  
117 consistent with fungal hyphae,[6,7] *Microsporidia* - small round hyper-reflective  
118 deposits (~ 2  $\mu\text{m}$ ) located in between keratocytes,[10] *Nocardia* - small branching  
119 filamentous structures within the corneal stroma,[9] and bacteria (control) – a mixture  
120 of inflammatory cells.

121

122

123

124 **Intra- and inter-observer agreement**

125 All digital images were assessed prospectively in the same standard fashion in the  
126 Reading Centre at Moorfields Eye Hospital by 4 observers (3 ophthalmologists and 1  
127 medical technician) with differing levels of experience in assessing keratitis on  
128 confocal microscopy as follows. Of the 3 ophthalmologists, observer A had 6 years of  
129 experience in assessing microbial keratitis with confocal microscopy, observer B, 10  
130 years of experience in confocal microscopy but not keratitis, and observer C, 6  
131 months of experience in assessing keratitis with confocal microscopy. Observer D  
132 was a medical technician who had 2 years of experience in performing confocal  
133 microscopy using the HRT II / RCM and analysing keratitis images but with no  
134 experience in the clinical appearance and treatment of different types of keratitis. To  
135 ensure each observer was familiar with the image appearance of different cell types  
136 obtained from the HRT II / RCM confocal microscope, examples of both normal  
137 cellular morphology and the standard images of different pathogens were shown in a  
138 presentation before their assessment. In addition, a series of five recent articles on  
139 diagnosing keratitis with the HRT II / RCM [4,5,7,9,10] were given to each observer  
140 to read 2 weeks prior to their scheduled assessment date.

141

142 The confocal images were viewed in random order and assigned an identification  
143 number from 1 to 62. To ensure that there was masking between observers, the order  
144 of viewing the images were randomised by computer before being assessed by the  
145 next observer on a different day. No clinical details regarding each case were made  
146 available to the observers. Each observer assessed the series of images in a masked  
147 fashion on slide show in Microsoft Powerpoint® and recorded the diagnosis  
148 corresponding to one of the following categories: AK, FK, *Microsporidia* (MK),

149 *Nocardia* (NK) or bacterial keratitis (BK). A reference sheet showing the range of  
150 sizes of resident and inflammatory cells including epithelium and macrophages, and  
151 pathogenic cells e.g. diameter of *Acanthamoeba* cysts was given to each observer.  
152 Intra-observer variability (repeatability) was evaluated by asking each observer to  
153 reassess the images, randomised in a different order, three weeks later in the same  
154 standard fashion. Inter-observer variability (reproducibility) was assessed by  
155 determining the level of agreement in diagnosis between observers. Readings of all  
156 the digital images were collected on a standard pro-forma and analysed.

157

## 158 **Data analysis**

159 Data analysis was performed with SPSS V14.0 (SPSS Inc, Chicago, USA). We  
160 calculated sensitivity, specificity, positive and negative likelihood ratios (LR) for both  
161 image set for each observer. Positive LR predicts the probability of a positive test  
162 result in patients with disease compared to those who do not have the disease.  
163 Negative LR predicts the probability of a negative test in those who have the disease  
164 compare to those who do not. The level of agreement between the reference standard  
165 and different observers, and both intra and inter-observer variability were determined  
166 using Kappa ( $\kappa$ ) statistic. The interpretation of  $\kappa$  statistic is as follows: 'poor' if  $\kappa \leq$   
167 0.20, 'fair' if  $\kappa$  0.21 – 0.40, 'moderate' if  $\kappa$  0.41 – 0.60, 'substantial' if  $\kappa$  0.61 – 0.80  
168 and 'good' if  $\kappa > 0.80$ . [16] In addition, we also calculated percentage agreement  
169 values between reference standard and observers, within-observers, and between  
170 different observers. Spearman's rank correlation coefficient ( $r_s$ ) was used to determine  
171 the relationship between the number of correct diagnoses and the duration of disease  
172 for AK, FK and BK respectively. The duration of disease was defined as the time  
173 from symptom onset to presentation to the Corneal and External Disease Service at

174 Moorfields. A value of  $P < 0.05$  was deemed statistically significant. MK and NK  
 175 were excluded from this analysis because the numbers were too small.

176

177 **RESULTS**

178

179 The reference standard consisted of 52 culture positive cases from corneal scrapings  
 180 and 10 histopathologically confirmed cases on corneal biopsy. Sensitivity, specificity  
 181 and likelihood ratio values for each observer are shown in Table 1.

**Table 1. Sensitivity, specificity and likelihood ratio values for each observer**

Observer	Sensitivity (95% CI)	Specificity (95% CI)	Positive LR (95% CI)	Negative LR (95% CI)
<b>A</b>				
1st assessment	55.8 (47.7-63.2)	57.9 (39.5-74.7)	1.32 (0.79-2.49)	0.76 (0.49-1.33)
2nd assessment	55.8 (47.6-61.8)	73.7 (55.0-87.3)	2.12 (1.06-4.87)	0.60 (0.44-0.95)
<b>B</b>				
1st assessment	53.4 (45.4-58.8)	78.9 (60.5-91.0)	2.53 (1.15-6.51)	0.59 (0.45-0.90)
2nd assessment	46.5 (38.6-50.9)	84.2 (66.4-94.2)	2.94 (1.15-8.82)	0.64 (0.52-0.93)
<b>C</b>				
1st assessment	27.9 (20.5-32.3)	84.2 (67.5-94.2)	1.77 (0.63-5.56)	0.86 (0.72-1.18)
2nd assessment	27.9 (20.4-33.2)	78.9 (61.9-90.9)	1.32 (0.54-3.66)	0.91 (0.74-1.29)
<b>D</b>				
1st assessment	44.2 (36.8-52.3)	42.1 (25.3-60.5)	0.76 (0.49-1.33)	1.33 (0.79-2.49)
2nd assessment	48.8 (40.7-56.3)	57.9 (39.6-74.8)	1.16 (0.67-2.23)	0.88 (0.59-1.50)

95% CI = 95% confidence intervals; LR = likelihood ratio

Positive LR = the probability of a positive test result in patients with disease compared to those who do not have the disease.

Negative LR = the probability of a negative test result in patients with disease compared to those who do not have the disease.

182

183 The highest sensitivity value obtained was 55.8% and the highest specificity value  
 184 84.2%. We found fair to moderate agreement between observers and reference  
 185 standard ( $\kappa$ , 0.22-0.44), moderate to good agreement in intra-observer variability ( $\kappa$ ,  
 186 0.56-0.88), and poor to moderate agreement in inter-observer variability ( $\kappa$ , 0.15-0.47),

187 Table 2. One observer (observer B) obtained the highest positive and lowest negative  
 188 LR for diagnosing microbial keratitis. This observer also achieved the best overall  
 189 kappa and percentage agreement values in diagnoses compared to reference standard,  
 190 Table 3. The best inter-observer agreement (percentage agreement, 61.3-66.1%;  $\kappa$ ,  
 191 0.43-0.47) was between observer A and B, the two most experienced observers in the  
 192 study. Observer C was the most repeatable (percentage agreement, 93.5%;  $\kappa$ , 0.88)  
 193 despite having the lowest kappa and percentage agreement values compared to  
 194 reference standard, Tables 2 &3.

195

**Table 2. Kappa values: reference standard versus observers, intra-observer and inter-observer variability**

Observer	Reference standard	A	B	C	D
A	0.36 (0.42)	0.56	0.43 (0.47)	0.23 (0.15)	0.28 (0.29)
B	0.44 (0.40)	0.43 (0.47)	0.76	0.40 (0.32)	0.18 (0.32)
C	0.24 (0.22)	0.23 (0.15)	0.40 (0.32)	0.88	0.24 (0.33)
D	0.27 (0.36)	0.28 (0.29)	0.18 (0.32)	0.24 (0.33)	0.65

Reference standard = culture or biopsy proven cases  
 Values in parentheses = observers' second assessment

196

197

**Table 3. Percentage (%) agreement values between reference standard and observers, within-observers, and between different observers.**

Observer	Reference standard	A	B	C	D
A	56.5 (61.3)	70.9	61.3 (66.1)	45.2 (43.5)	45.2 (50)
B	61.3 (58.1)	61.3 (66.1)	85.5	64.5 (61.3)	37.1 (53.2)
C	45.2 (43.5)	45.2 (43.5)	64.5 (61.3)	93.5	40.3 (53.2)
D	43.5 (51.6)	45.2 (50)	37.1 (53.2)	40.3 (53.2)	72.6

Reference standard = culture or biopsy proven cases  
 Values in parentheses = observers' second assessment

198

199

200 Complete agreement in diagnosis between all the observers and reference standard for  
 201 both assessments were found in 3/26 (11.5%) cases of AK, 8/19 (42.1%) cases of BK,  
 202 and 1/12 (8.3%) case of FK. In contrast, none of the observers identified  
 203 *Acanthamoeba* in 5/26 (19.2%) cases, fungus in 4/12 (33.3%), and confused BK with  
 204 other diagnoses in 2/19 (10.5%) cases. Observer B was the only one who managed to  
 205 diagnose NK correctly in one case. The percentage correct diagnosis for the different  
 206 types of keratitis is shown in Table 4. A breakdown of all the diagnoses for each  
 207 observer for the different keratitis category is shown in the appendix.  
 208

**Table 4. Percentage of correct diagnoses of the different causes of keratitis for different observers**

Reference standard Diagnosis	Observer, n (%)			
	Observer A	Observer B	Observer C	Observer D
<i>Acanthamoeba</i> (n=26)	19 (73.1)	11 (42.3)	4 (15.4)	12 (46.2)
<b>Bacteria</b> * (n=19)	10 (52.6)	15 (78.9)	15 (78.9)	8 (42.1)
<b>Fungus</b> † (n=12)	1 (8.3)	5 (41.2)	5 (41.2)	5 (41.2)
<i>Microsporidia</i> (n=3)	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)
<i>Nocardia</i> (n=2)	0	1 (50)	0	0

Percentage correct diagnosis was calculated by taking into account only those cases where each observer had made the correct diagnosis on both assessments.

\* 2 *Enterobacter*, 1 *Haemophilus*, 1 *Moraxella*, 1, *Mycobacteria*, 1 *Pasteurella*, 3 *Pseudomonas*, 1 *Serratia*, 7 *Staphylococcus*, 2 *Streptococcus*.

† 1 *Alternaria*, 1 *Candida*, 10 *Fusarium*.

209

210 Figure 1 shows a series of images demonstrating the difference in appearance between  
 211 correctly diagnosed versus incorrectly diagnosed cases. Figures 1 A and F show a  
 212 case of late diagnosed AK versus early diagnosed AK; note the presence of  
 213 inflammatory cells in the epithelium in early AK making distinction between host  
 214 cells and *Acanthamoeba* cysts and trophozoites difficult, whereas in delayed  
 215 diagnosed AK, single or clusters of cysts were seen in the stroma with minimal host  
 216 immune and resident cells seen. The incorrectly diagnosed cases demonstrate the

217 difficulties in distinguishing host cells from pathogenic organisms, and *Nocardia* (Fig  
218 1J) from FK because of their similarity in appearance on confocal microscopy.

219

220 Figure 2 shows a plot between the number of correct diagnoses for Acanthamoeba,  
221 bacteria and fungal keratitis versus the duration of disease (days). The graph shows a  
222 moderate correlation between the number of correctly diagnosed cases and the  
223 duration of disease for AK ( $r_s = 0.60$ ,  $p = 0.001$ ), but not for BK ( $r_s = 0.17$ ,  $P = 0.49$ ) or  
224 FK ( $r_s = -0.19$ ,  $p = 0.57$ ), respectively. Therefore, the longer the duration of AK, the  
225 higher the likelihood that a correct diagnosis was made by the observers in grading  
226 the confocal images.

227

## 228 **DISCUSSION**

229

230 Acanthamoeba and fungus are uncommon causes of corneal infection for which early  
231 diagnosis is paramount because it yields better prognosis and reduces ocular  
232 morbidity.[17,18] Although the current reference standard for diagnosing microbial  
233 keratitis is corneal culture, the sensitivity varies because of numerous factors.[19]

234

235 The HRT II / RCM *in vivo* confocal microscope has been shown to be useful in  
236 diagnosing a range of pathogens but validation studies of this new technology are few.

237 A recent review has reported the efficacy of diagnosing infections keratitis with  
238 confocal microscopy to be inconclusive, with the possible exception of AK.[20] Our  
239 results show moderate sensitivity and moderate to high specificity values in  
240 diagnosing microbial keratitis with the HRT II / RCM confocal microscope, whereas



241 both Kanavi et al[11] and Tu et al[12] found very high sensitivity (>90%) in  
242 diagnosing AK and FK respectively with the Confoscan 3. Tu et al,[12] using multi-  
243 test referencing standards, reported that when there are both clinical characteristics  
244 and objective evidence of AK, the adjunctive usage of confocal microscopy exhibited  
245 a sensitivity of 90.6% and specificity of 100%. In our study, we set out to evaluate the  
246 diagnostic accuracy of confocal microscopy as a stand alone tool rather than a  
247 supportive investigative technique, without the bias and influence of clinical findings.  
248 Although assessing confocal images in the absence of clinical data does not reflect the  
249 use of confocal microscopy in clinical settings, it is the only way to avoid bias when  
250 analysing the images. Our inclusion criteria were based on culture positive cases  
251 irrespective of confocal classification. Although we chose only one representative  
252 image from each case this was the best available image for the organism that was  
253 cultured from each case giving the observers the best opportunity to make a correct  
254 confocal diagnosis; we believe that reviewing a series of images from each case  
255 would either have made a correct confocal diagnosis more difficult or have had no  
256 effect on the outcome. In addition, it allowed standardisation when viewing the  
257 images so that all observers assessed the same number of images consecutively. The  
258 absence of controls in the previous studies and the use of confocal ‘positive’ without  
259 culture confirmation as a reference standard, or for the case definition[11,12], could  
260 lead to selection bias and misdiagnosis resulting in an overestimation of sensitivity  
261 values.[12,21,22] This is evident from our controls in which immune cells can often  
262 be confused with AK cysts and vice versa leading to erroneous diagnosis.  
263 Furthermore, ‘good’ confocal images have been illustrated in most published studies  
264 to present findings without discussion of difficulties in analysing equivocal images.  
265 We found fair to moderate agreement between reference standard and observer

266 diagnosis when a case mix of equivocal and unequivocal images were analysed by our  
267 observers. The rigorous criteria in our study design in regard to the use of masked  
268 observers and controls could explain why sensitivity values, even for the most  
269 experienced observer, were lower.

270

271 Another explanation for the very high sensitivity values reported in one previous  
272 study was the use of only one ophthalmology trained observer who, in addition to  
273 being unmasked to the clinical findings, was experienced in the use of confocal  
274 microscopy for keratitis diagnosis: this makes it difficult to extrapolate the results to  
275 what might be expected outside their centres.[12] To evaluate the potential of using  
276 this technology in clinics where an ophthalmologist with experience in confocal  
277 microscopy may not be available, our graders included 2 experienced  
278 ophthalmologists, an inexperienced ophthalmologist and an experienced technician.  
279 We found a two-fold difference in sensitivity between the most experienced and the  
280 least experienced observer indicating higher diagnostic accuracy with clinicians  
281 experienced in confocal microscopy. Our results indicate the sensitivity value with a  
282 trained technician, with no experience in the clinical appearance of different types of  
283 microbial keratitis, was better than an inexperienced medical observer but with a  
284 lower specificity value and positive LR. This raises the possibility of training non-  
285 medical personnel, in performing and analysing keratitis images. The highest positive  
286 LR and lowest negative LR was achieved by observer B who was experienced in  
287 confocal imaging of normal corneal anatomy and various pathological conditions  
288 other than microbial keratitis, indicating experience gained in other aspects of  
289 confocal microscopy improves the diagnostic outcome.

290

291 Intra-observer agreement (repeatability) was found to be moderate to good, indicative  
292 of good observer repeatability in grading the images irrespective of the accuracy of  
293 their diagnoses. Observer experience did not appear to improve intra-observer  
294 repeatability as the observer with the lowest sensitivity had the highest repeatability  
295 and vice versa. Inter-observer agreement (reproducibility) was poor to moderate  
296 between different observers because of factors such as observer experience and  
297 differences in techniques of classifying images by different observers. The two  
298 observers who had the highest sensitivity values also had the best inter-observer  
299 reproducibility, indicating experienced observers achieved a higher diagnostic  
300 accuracy and reproducibility than less experienced observers. Therefore, to improve  
301 reliability the same experienced operator should be employed if sequential imaging of  
302 a patient is required.

303

304 Our observers were able to diagnose AK more accurately than any other type of  
305 keratitis. The unique appearance of *Acanthamoeba* cysts on confocal microscopy and  
306 the higher number of AK compared to other conditions in our study might explain this  
307 outcome. However, AK was commonly confused with controls and vice versa because  
308 of the diagnostic difficulty with some of the equivocal images. There was a marked  
309 association between the accuracy of diagnosing AK and the duration of disease.  
310 Previous case reports have mainly described the morphological features of cysts and  
311 trophozoites in the epithelium and stroma during active infection,[4,5] but have not  
312 related the number of cysts seen and the way they distribute with the different stages  
313 of the disease process. In early disease, where the organism is mainly confined to the  
314 epithelium, the presence of large numbers of inflammatory cells made diagnosing AK  
315 more difficult because of the difficulty in distinguishing AK cysts and particularly

316 trophozoites from inflammatory cells.[5] Late presentation was associated with either  
317 a greater number of *Acanthamoeba* cysts seen in the images or the fact that they were  
318 easier to identify because of a reduction in the type and number of host cells seen. Our  
319 experience, therefore, suggests that AK is easier to identify with confocal microscopy  
320 in the later stages of infection.

321

322 The use of confocal microscopy in diagnosing FK has been widely reported in the  
323 literature.[6-8] Filamentous fungal hyphae have characteristic linear hyper-reflective  
324 lesions branching at 45 or 90 degrees angle,[7] whereas candida infection produces  
325 pseudofilaments.[7] Despite these well described confocal appearances of FK in the  
326 literature, the percentage of correct diagnosis in our series was low possibly due to  
327 difficulties in differentiating other linear images from fungal hyphae.[23]

328

329 *Nocardia* and *Microsporidia* species are rare causes of microbial keratitis.[24]  
330 Clinically, *Nocardia* may be misdiagnosed as mycotic or mycobacterial keratitis,[9,25]  
331 whilst *Microsporidia* can be misdiagnosed as AK or herpes simplex keratitis. Despite  
332 the rarity of these organisms, because of the unique appearance on confocal  
333 microscopy with *Microsporidia*,[10] two observers managed to identify this organism  
334 correctly in both of their assessments. Only observer B managed to obtain the correct  
335 diagnosis in both assessments for diagnosing one case of *Nocardia* keratitis; the  
336 unfamiliarity in interpreting confocal images of *Nocardia*, the similarity in appearance  
337 of fungal hyphae and *Nocardia* filaments, and the small number of cases in our study  
338 made diagnosing this organism difficult. The inclusion of both *Nocardia* and  
339 *Microsporidia* cases might have reduced the overall sensitivity and specificity values

340 but as confocal findings of both organisms have been reported, we believe it was  
341 appropriate to include them in the study.

342

343 In summary, to the best of our knowledge, this is the first study evaluating the  
344 diagnostic accuracy of microbial keratitis using a single reference standard for  
345 different masked observers with the HRT II / RCM confocal microscope. Although  
346 confocal microscopy is non-invasive and can provide a rapid diagnosis for microbial  
347 keratitis, (i) similarities between inflammatory and pathogenic cells, and (ii) difficulty  
348 in interpreting equivocal images, limits its usefulness as a stand-alone tool in  
349 diagnosing keratitis. Confocal microscopy is a useful adjunct in managing refractory  
350 cases and we have shown that the diagnostic accuracy improves with clinician  
351 experience. However, the diagnostic accuracy of confocal microscopy used in  
352 isolation from the clinical assessment is still too low to be a substitute for tissue  
353 diagnosis, particularly in patients with progressive disease. Improvement in clinician  
354 training and experience, greater standardization of image interpretation, and the  
355 development of new software in tandem with higher resolution imaging is likely to  
356 improve the diagnostic accuracy of this technology in diagnosing microbial keratitis  
357 in the future.

358

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361

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363 None declared

364

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367

368

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465 **FIGURE LEGENDS**

466

467 **Figure 1**

468

469 Confocal scans of correctly diagnosed versus incorrectly diagnosed cases.

470 Figures A to D demonstrate the characteristic features of inflammatory cells and

471 pathogenic organisms on confocal microscopy in which all the observers had made

472 the correct diagnoses. A, *Acanthamoeba* cysts (white arrow), some with double-

473 walled appearance (dotted white arrow). B, Inflammatory cells (black arrows). C,

474 Fungal hyphae (black arrows). D, *Microsporidia* organisms (white arrows).

475

476 Figures E to J show a series of images of incorrectly diagnosed cases demonstrating

477 the difficulty in distinguishing host cells from pathogenic organisms and *Nocardia*

478 from fungal keratitis. E, Shows *Nocardia* filaments (white arrows) - only observer B

479 identified this correctly with all the other observers graded it as fungal hyphae. F,

480 Cultured *Acanthamoeba* - misdiagnosed as bacterial keratitis by observers B, C and D;

481 possible *Acanthamoeba* cysts (white arrows), and possible inflammatory cells (black

482 arrows). G, Cultured bacteria (*Staphylococcus aureus*) but was misdiagnosed as

483 *Acanthamoeba* by all the observers; multiple round lesions that could be identified as

484 inflammatory or *Acanthamoeba* cysts (white arrows). H, Cultured bacteria -

485 diagnosed as fungal keratitis by observers C & D; linear hyphae-like opacities that

486 were confused with fungal hyphae (white arrows). I, Cultured bacteria - diagnosed as

487 *Microsporidia* by all the observers; small hyper-reflective granules that appear similar

488 to *Microsporidia* organisms (white arrows). J, Cultured positive for *Alternaria* and

489 *Staphylococcus aureus* but diagnosed as *Nocardia* by observers A, B and D; hyphae  
490 type lesions that appear similar to *Nocardia* filaments (black arrows).

491

492 **Figure 2**

493

494 Scattered plot showing the relationship between number of correctly diagnosed cases  
495 and duration of disease (days) for *Acanthamoeba*, bacteria and fungal keratitis.

496



